B cell development in fetal liver and adult bone marrow

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D i s s e r t a t i o n

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1. Introduction

1.1. Fundamental features of the immune system

We spend our lives surrounded by potentially pathogenic microorganisms, nevertheless we only rarely become ill. This is due to the highly effective immune system that has evolved in vertebrates. Corresponding to the immense number of pathogens and various ways of infection, the immune system is composed of a great variety of cells as well as messenger and effector molecules. In principle, two systems of defense are distinguished: the innate and the adaptive immune system. The innate immune system represents a first line of defense, and protects the organism against many common bacterial infections at an early time point with its rather unspecific mechanisms and invariant receptors, recognizing common features of pathogens such as bacterial cell wall components or double-stranded RNA. This system is so effective that we often do not notice infections by pathogens of low virulence. But the innate immune system can not always eliminate infectious organisms, and there are also many pathogens which can evade the mechanisms of the innate immune system. The cellular members of the adaptive immune system act much more specific. They possess diverse surface receptors and can recognize and target pathogenic microorganisms or cells infected by them. In addition, they mediate – sometimes lifelong – protection against re-infection by the same pathogen due to an immunological memory. The synergistic action of both the innate and the adaptive immune system is the basis for a rapid, highly specific and protective immune response against potentially pathogenic microorganisms. This allows survival in a principally hostile microbial environment.

1.2. The adaptive immune system

The two cellular mediators of adaptive immunity, the T and the B lymphocytes, express on their surface highly diverse receptors capable of recognizing a wide diversity of antigens, i.e. molecules or structures, that induce an immune response. Each individual lymphocyte clone bears receptors of a single antigen specificity only.

The T lymphocytes, also named T cells, are responsible for cellular immunity. T cells, like other blood cells, are known to be derived from hematopoietic stem cells. T cell progenitors migrate from the fetal liver (FL) or after birth from the bone marrow (BM) to the thymus,

where they mature (Kruisbeek, 1999). After maturation, they leave the thymus and recirculate through the secondary lymphatic organs, e.g. spleen, lymph nodes and Peyer's patches.

T lymphocytes express the T-cell receptor (TCR) as antigen-binding structure on their surface. For the majority of T cells the TCR is a heterodimer consisting of an α and a β chain (α:β T cells). Such T cells are not able to recognize native antigen, but only a peptide fragment bound to a molecule of the major histocompatibility complex (MHC). Two classes of the MHC molecules exist: MHC class I molecules, that mainly present peptides of the cytosolic compartment, and MHC class II molecules, which primarily present peptides of proteins that are taken up via endocytic vesicles (Watts and Powis, 1999).

Based on cell surface markers and functional properties the α:β T cells can be subdivided into at least two subsets. First, into the cytotoxic T cells that display the surface antigen CD8 and second, into T-helper cells, characterized by the surface antigen CD4. The $CD8^+$ cytotoxic T cells can recognize, for instance, virus infected cells via antigens presented by MHC class I and then kill these cells by induction of apoptosis, thus stopping further spreading of the virus. The $CD4^+$ T-helper cells recognize peptide fragments presented by MHC class II molecules and are specialized activators for other cells. As T_H1 cells they activate macrophages to kill pathogens (Paulnock, 1992), and as T_H2 cells they activate B lymphocytes to differentiate and to produce antibodies (Parker, 1993).

Some T cells bear an alternative form of the TCR with γ and δ chains (γ:δ T cells). The function of these γ:δ T cells are at present still unknown, likewise their ligands. Interestingly, it has been shown that the γ:δ receptor is sometimes able to recognize antigen directly (Born *et al.*, 1999).

B lymphocytes (or B cells) are the cells responsible for the humoral immunity. On their surface, they express immunoglobulins (Ig) as antigen receptors (B-cell receptor, BCR), which can recognize native antigen. After antigen contact and further activation, B cells can differentiate into plasma cells and secrete the immunoglobulins as antibodies. These antibodies participate in three different ways in the combat against an infection. First, they bind bacterial toxins and viruses and thereby block their access to cells that they may infect or destroy (neutralization). Second, antibodies coat pathogens and foreign particles (opsonization), providing a signal for their elimination by phagocytic cells of the innate immune system (like macrophages or neutrophils). The third function upon antigen-binding is the activation of a system of plasma proteins known as complement, leading to the destruction and removal of the pathogen.

Like T lymphocytes and other blood cells, B lymphocytes are derived from hematopoietic stem cells. In mammals B cells both develop and mature in fetal liver or after birth in bone marrow (Akashi *et al.*, 2000a; Busslinger *et al.*, 2000). In birds they require the bursa of Fabricius for final maturation, which was the reason to originally name these cells B lymphocytes.

Based on cell surface markers, anatomical localization and function, mature B cells are classified into at least two subsets, B1 and B2. The B1 cells dominate the body cavities, like pleural cavity and peritoneum, while they can be found relatively rarely in the peripheral lymphoid organs, like spleen and lymph nodes. B1 cells often produce poly-reactive antibodies against bacterial polysaccharides, which are probably important for an early immune response against bacterial pathogens (Boes *et al.*, 1998). The B2 cells, also called conventional B cells, play an essential role in the adaptive immune response with their monospecific antibodies. B2 cells represent the predominating B-cell population in the peripheral lymphoid organs, like spleen and lymph nodes, but they can also be found in body cavities.

The major difference between the two populations is that B2 cells are not generated before birth. Without antigen contact they have only a limited life span and are continuously replaced by newly formed B cells from the bone marrow. This is in contrast to the B1 cells, which possess the capacity of self-renewal, and it has been suggested that their generation is completed shortly after birth. Presumably, they originate mainly in the fetal liver and neonatal bone marrow (Herzenberg, 2000; Hayakawa and Hardy, 2000).

1.3. Structure of an antibody molecule

The effector molecules secreted by activated B cells are the antibodies. Antibodies are Y-shaped molecules composed of two identical heavy (H) and two identical light (L) polypeptide chains normally linked by disulfide bonds (Fig. 1-1). Each chain consists of a variable and a constant region. The variable (V) region at the amino-terminal end displays three regions with particular variability, the so called hypervariable or complementarity-determining regions (CDR), surrounded by four regions which are relatively invariant, named framework regions (FWR). While the FWRs are important for the structure of the antibody molecule, the CDRs of both the heavy and the light chain together form the antigen-binding site of the antibody.

The constant (C) carboxy-terminal part of the heavy chain determines the class or isotype of the antibody. There are five main heavy-chain classes: IgM, IgD, IgG, IgE and IgA, where the IgG-isotype in mice is subdivided into the subclasses IgG1, IgG2a, IgG2b and IgG3. The corresponding heavy chains are denoted by Greek letters: μ, δ, γ, ε and α, and γ_1, γ_{2a}, γ_{2b} and γ_3 respectively. The class of an antibody molecule determines its functional activity. IgG antibodies activate phagocytic cells, while IgE antibodies stimulate mast cells to release histamine.

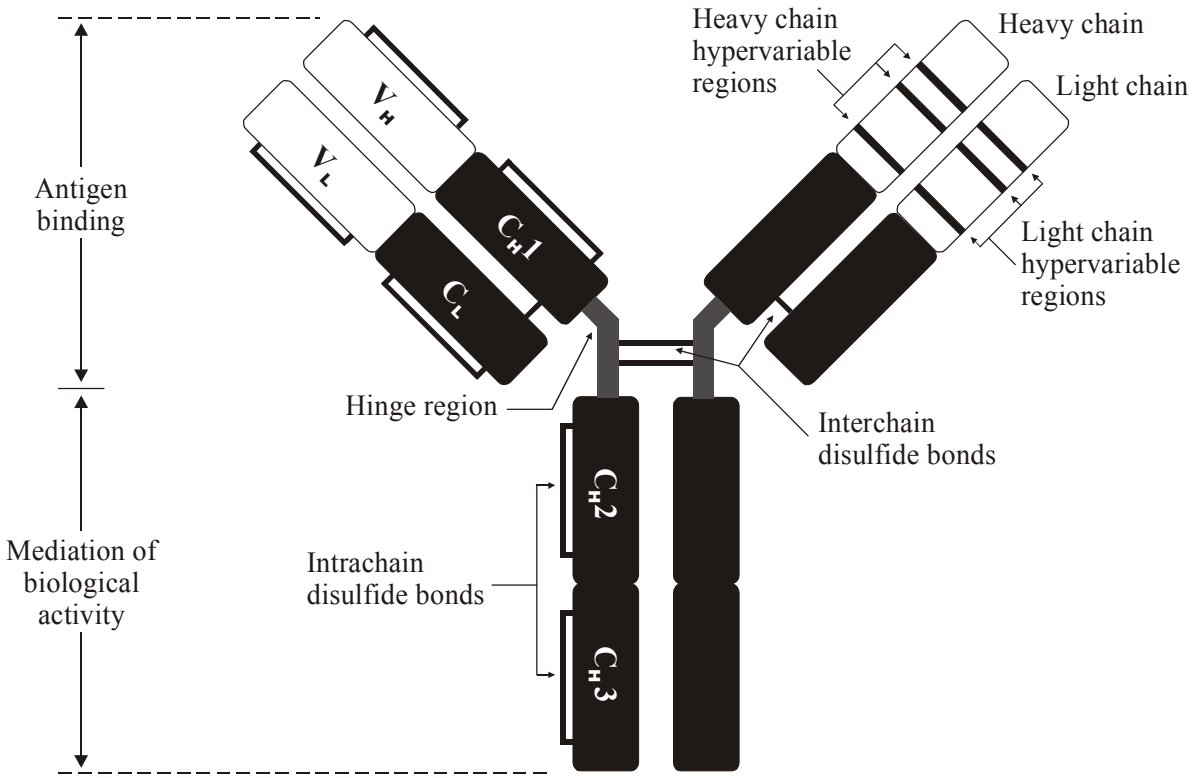


Fig. 1-1: Structure of an antibody molecule. Shown are the functional domains, intrachain and interchain disulfide bonds and the hypervariable regions of both the heavy and the light chains of an IgG1 molecule.

Two different types of light chains exist: kappa (κ) and lambda (λ). No functional difference has been found between antibodies bearing κ or λ light chains, only the ratio of the two types is characteristic for a species. In humans the κ to λ ratio is about 2 : 1, in mice 10 : 1.

Each antibody molecule is expressed either in a membrane-bound form or as secreted molecule, except IgD, which exists only in the membrane-bound form. The membrane-bound

antibody molecule is anchored by a hydrophobic transmembrane domain in the membrane of B lymphocytes and is called BCR. The BCR normally contains only a small cytoplasmic domain, thus can not transduce signals by itself. It is therefore complexed with the signal transducing molecules Igα and Igβ (Reth *et al.*, 2000).

In the secreted form, a secretory tail replaces the transmembrane domain and the cytoplasmic component. Membrane-bound antibodies like IgG and IgE molecules exist only as monomers, while secreted IgM and IgA antibodies are usually synthesized as multimers. IgA forms dimers, whereas IgM forms pentamers or hexamers (Johansen *et al.*, 2000). In these multimers the single monomeric subunits are joined by disulfide bonds and an additional polypeptide chain, the J (joining) chain (summarized in Frazer and Capra, 1999).

1.4. Generation of the primary antibody repertoire

An organism must be able to recognize each potential antigen or to develop a specific antibody against each particular antigen. The multitude of potential antibodies is formed from a very limited number of genes that encode them. The great diversity of the antibodies is only realized by the fact that genes encoding the variable regions of the antibody molecules are composed of several gene segments, which are separated in the germline but combined in developing B lymphocytes in a process called somatic recombination or rearrangement. A similar process takes place during receptor formation in developing T lymphocytes.

Each genetic locus encoding a particular immunoglobulin chain is special concerning organization and arrangement of the gene segments (summarized in Tonegawa, 1983; Max, 1999). Moreover, they are also localized on different chromosomes. The heavy chain locus of the mouse is on chromosome 12, while the κ and the λ light chain loci are on chromosome 6 and 16, respectively.

1.4.1. The heavy chain locus

The heavy chain variable regions are encoded in three gene segments: V_H, D_H and J_H. The region containing these gene segments is very complex and comprises hundred or more V_H (variable) gene segments, the exact number of them is not yet known. By means of sequence homology, different V_H gene segments could be classified into 14 V_H gene families. A considerable number of these V_H gene segments are not able to rearrange or lead to non-

functional polypeptide chains due to mutations. They are therefore called pseudo-genes. Downstream of the V_H gene segments a cluster of 16 functional D_H (diversity) segments can be found (Fig. 1-2). Localized further 3' are four functional J_H (joining) segments.

The particular gene segments are flanked by so called recombination signal sequences (RSS), which are essential for the site-directed recombination process. A RSS contains a highly conserved palindromic heptamer and a moderately well conserved A/T rich nonamer sequence, which are separated by either 12 or 23 base pairs (bp) of non-conserved spacer DNA (Fig. 1-3). While the V_H and J_H gene segments are flanked by RSS with a 23 bp spacer, the D_H segments are flanked at both sides by RSS with 12 bp spacers. Recombination can combine only a gene segment flanked by a 12mer-spaced RSS with one displaying a 23mer-spaced RSS (the 12/23 rule, summarized in Tonegawa, 1983; Gellert, 1997). By this means, it is guaranteed that a V_H gene segment is always joined to a J_H gene segment via an intermediate D_H gene segment.

The immunoglobulin C_H region gene segments form a large cluster 3' of the J_H gene segments. Each C_H region gene coding for the constant region of the different antibody classes and subclasses is split into separate exons corresponding to the domains of an antibody molecule.

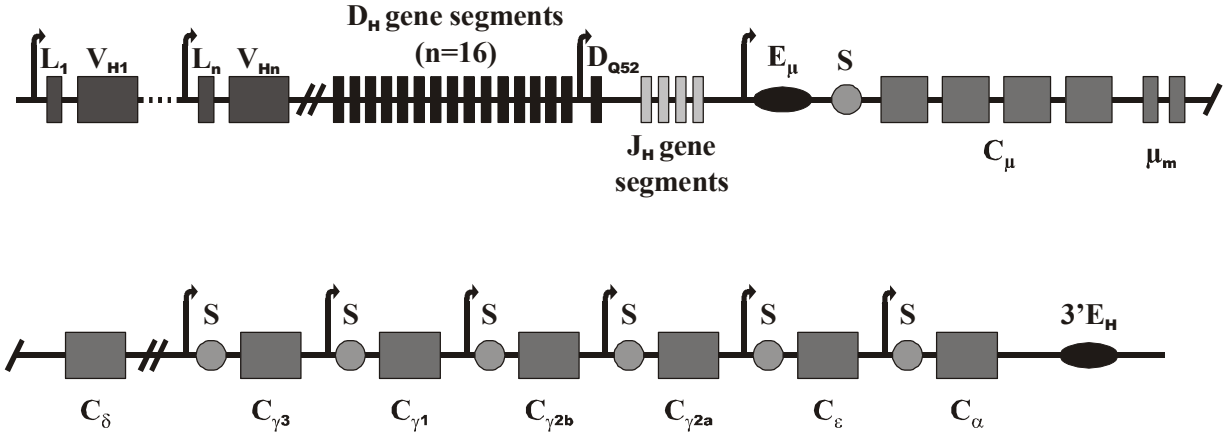


Fig. 1-2: The murine heavy chain locus. The gene segments are displayed in germline configuration (not to scale). Each V_H gene segment is flanked by a leader (L) region, coding for a leader peptide important for the intracellular transport into the endoplasmatic reticulum. After the transport the leader peptide is cleaved off. Shown are the intron (E_μ) and the 3' enhancer (3'E_μ). Arrows indicate promoters. Only for C_μ the four exons in addition to the two short exons coding for the transmembrane and cytoplasmic part are shown. S: switch region.

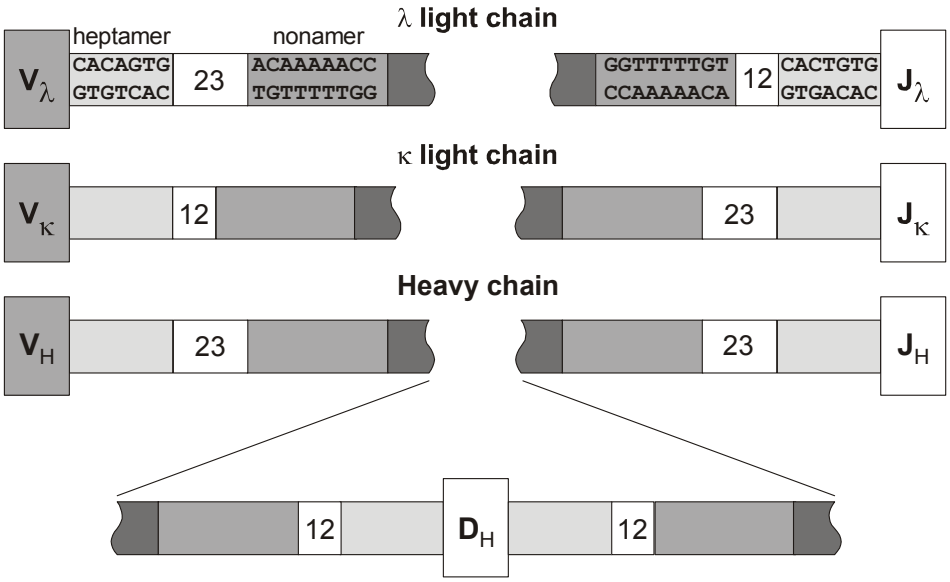


Fig. 1-3: Flanking recombination signal sequences of heavy and light chains. Shown are the conserved heptamer and nonamer sequences as well as the spacer length of the particular gene segments.

After maturation and exit from the bone marrow, B lymphocytes that had not encountered antigen yet display two immunoglobulin isotypes with identical V regions at their surface, IgM and IgD. Mechanistically, these cells produce a long primary transcript including both the C_μ and the C_δ gene segment, that resides downstream of Cμ. Alternative processing leads to two different mRNA molecules and consequently to the co-expression of IgM or IgD. A similar mRNA modification takes place after antigen stimulation, when the antibody is expressed in its secreted form instead of the membrane-bound form.

After antigen contact B cells are also able to express other antibody classes. In a reaction called class switch recombination (CSR), the original C_H gene segment is replaced by another downstream segment changing the antibody class and effector function. The antigen specificity remains the same during these events.

1.4.2. The κ light chain locus

The variable regions of light chains are encoded by V and J gene segments only. For the κ locus so far 140 V_κ gene segments are localized and sequenced. 75 of them are functional,

while 44 are pseudo-genes and 21 are only potentially functional. The latter bear mutations in promoters, splice-sites, RSS or exons, but nevertheless a cDNA could be found in the database (Roschenthaler *et al.*, 2000). A unique characteristic of the κ locus is, that the $V_κ$ gene segments are located in both transcriptional orientations relative to the C region.

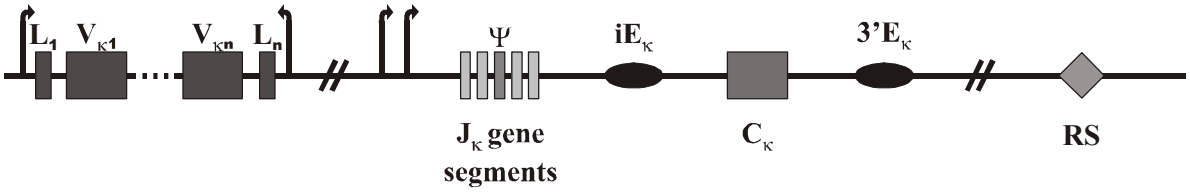


Fig. 1-4: The murine κ light chain locus. The gene segments are displayed in germline configuration (not to scale). Both possible transcriptional orientations of the $V_κ$ segments are indicated by the position of the leader regions. Shown are the intron ($iE_κ$) and the 3' enhancer ($3'E_κ$). Promoters are indicated by arrows, pseudo-genes by the Greek letter Ψ. RS: recombining sequence.

Downstream of the $V_κ$ gene segments is a cluster of five $J_κ$ genes (Fig. 1-4). One of them ($J_κ3$) was found to be non-functional. The single segments are flanked 5' with 23mer-spaced RSS whereas consequently the $V_κ$ gene segments are 3' flanked by 12mer-spaced RSS. Separated by a 2.5 kb intron from the $J_κ$ gene segments, a single $C_κ$ segment could be found (Max *et al.*, 1981).

Similar to the V_H gene segments it is also possible to subdivide the $V_κ$ gene segments into 18 different families by means of sequence homology. The biggest family $V_κ04/05$ contains 33 $V_κ$ gene segments whereas other families like $V_κ22$, $V_κdv$, $V_κ38c$ and $V_κRF$ consist only of a single member. The $V_κ21$ genes form the most 3' or most J-proximal family, whereas $V_κ02$ is a family at the 5' end and could be taken as the J-distal family (Fig. 1-5).

About 25 kb 3' of the $C_κ$ gene segment a recombining sequence (RS; Muller *et al.*, 1990) without coding function can be found (Daitch *et al.*, 1992). This RS could rearrange with a $V_κ$ gene segment leading to the deletion of the intervening segments. This results in inactivation of the complete κ locus on this chromosome because further rearrangements are excluded this way (Durdik *et al.*, 1984; Moore *et al.*, 1985). Initially it was supposed that this recombination plays a role in the regulation of the λ light chain locus, but later examinations

argued against it (Dunda and Corcos, 1997; Engel *et al.*, 1999). Involvement in receptor-editing was postulated instead (Nemazee, 2000).

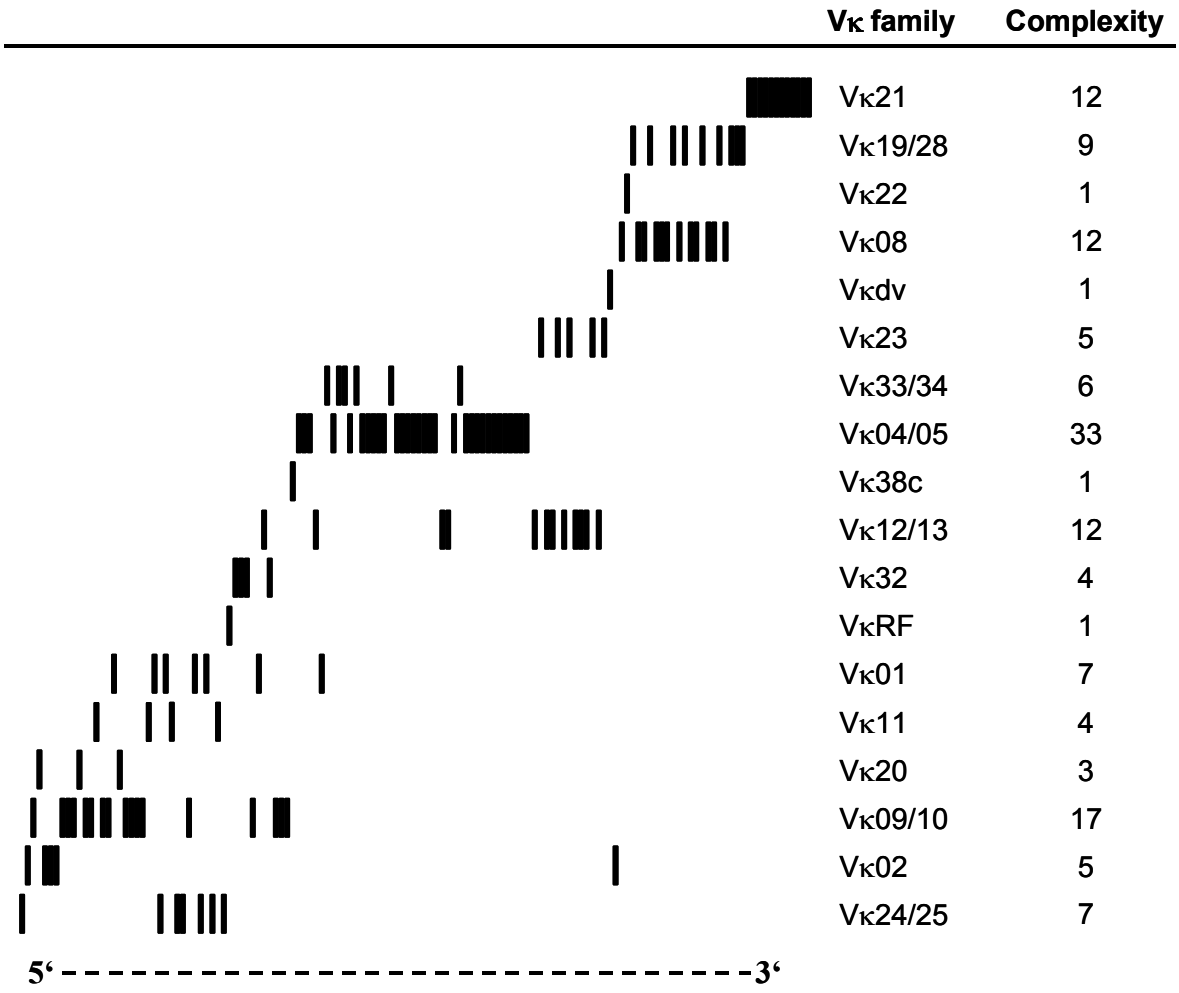


Fig. 1-5: Map of the murine Vκ gene locus. The complexity and the relative chromosomal position of the different V_κ gene families are shown. Each bar represents a single V_κ gene segment. Two genes of the V_κ04/05 family are not included in this map, because the exact localization was not determined so far.

1.4.3. The λ light chain locus

In contrast to both the heavy and the κ light chain locus the J and C segments of the λ locus are arranged in pairs (Fig. 1-6). Three of the four $J_\lambda C_\lambda$ clusters are functional. Only three V_λ genes are present in the λ locus of laboratory mouse strains tested so far. One of these V_λ genes ($V_\lambda 1$) is located between the two $J_\lambda C_\lambda$ clusters thus most likely excluding rearrangements with the upstream $J_\lambda C_\lambda$ cluster. The V_λ gene segments are flanked by 23mer-

spaced RSS and the J_λ segments by 12mer-spaced RSS in agreement with the 12/23 rule. The organization of the λ locus seems to be the result of two sequential gene duplication events (Blomberg and Tonegawa, 1982; Selsing *et al.*, 1982). The second V_λ downstream of $V_\lambda 1$ was obviously lost after this event.

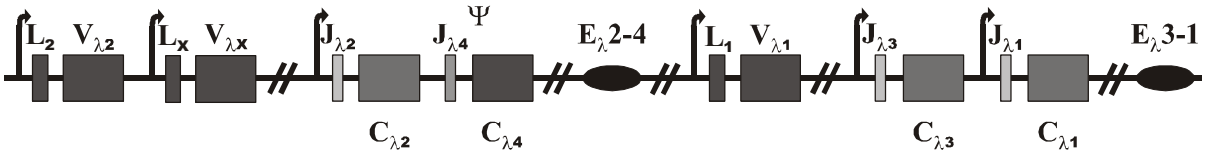


Fig. 1-6: The murine λ light chain locus. The gene segments are displayed in germline configuration (not to scale). Shown are the enhancers $E_\lambda 2\text{-}4$ and $E_\lambda 3\text{-}1$. Promoters are indicated by arrows, pseudo-genes by the Greek letter Ψ.

1.4.4. The recombination activating genes *Rag1* and *Rag2*

The gene products of the recombination activating genes *Rag1* and *Rag2*, the proteins Rag1 and Rag2, play an essential role in the recombination process. This was demonstrated in mice in which *Rag1* (Mombaerts *et al.*, 1992) or *Rag2* (Shinkai *et al.*, 1992) was inactivated by homologous recombination. These mice can not rearrange their antigen-receptor genes, thus leading to a lack of all B and T lymphocytes.

The recombination activating genes *Rag1* and *Rag2* are located in mice on chromosome 2 in a tail-to-tail configuration separated by a 7 kb intergenic region (Fig. 1-7; Bertrand *et al.*, 1998). *Rag1* is composed of two exons, while *Rag2* consists of three, whereby the entire coding sequence (CDS) in both cases is restricted to the last exon. The Rag2 mRNA, 2.1 kb in length, encodes a polypeptide of 527 amino acids with a molecular size of 58 kD (Oettinger *et al.*, 1990) whereas the Rag1 mRNA is 6.6 kb in length and encodes a polypeptide of 1040 amino acids with a molecular size of 119 kD (Schatz *et al.*, 1989). Both genes show no homology to each other, but in all species examined so far, the genomic organization of the Rag locus was conserved, with the two genes tightly linked and convergently transcribed (Schatz *et al.*, 1992). This observation led to the hypothesis that the *Rag* genes were derived from an ancestral transposon that integrated into an early vertebrate genome (Oettinger *et al.*, 1990; Thompson, 1995). With one exception, the coding region of each gene is contained within a single exon (Hansen and Kaattari, 1995).

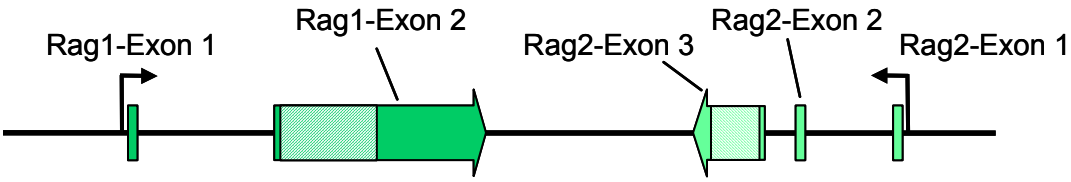


Fig. 1-7: The murine Rag locus. Shown are the exons of *Rag1* and *Rag2*. Both exons 1 and *Rag2*-exon 2 are not to scale, they are only about 70 nucleotides long. Hatched regions represent the coding sequence. Promoters and transcriptional orientation are indicated by arrows.

Rag gene expression is restricted to the lymphocyte lineages, although low levels of *Rag1* transcripts have been reported in non-lymphoid tissues (Chun *et al.*, 1991; Hayakawa *et al.*, 1996). Usually expression is found in developing B and T lymphocytes and ceases upon maturation. In addition, it was claimed that the Rag locus could be reactivated in mouse germinal center B cells (Han *et al.*, 1996; Hikida *et al.*, 1996; Papavasiliou *et al.*, 1997; Hikida and Ohmori, 1998). However, additional studies with transgenic or knock-in *Rag* reporter constructs indicated that *Rag* expression can not be re-induced in most mature B lineage cells and the vast majority of *Rag* expressing cells represent B cell progenitors or precursors (Yu *et al.*, 1999b; Monroe *et al.*, 1999b).

The mechanisms responsible for the lymphoid- and developmental stage-specific regulation of the *Rag* genes are poorly understood. Only the murine *Rag2* promoter shows tissue specificity (Monroe *et al.*, 1999a; Lauring and Schlissel, 1999), while ubiquitous basal activity is found for both the murine and the human *Rag1* as well as for the human *Rag2* promoter (Fuller and Storb, 1997; Kurioka *et al.*, 1996; Zarrin *et al.*, 1997). Additional sequences 5' of the *Rag2* gene appear to regulate both *Rag1* and *Rag2*, an organization that has been suggested to support the notion that the two genes were placed into the genome via a primordial transposon (Yu *et al.*, 1999a). Recently, a B-cell specific and evolutionarily conserved transcriptional enhancer in the Rag locus, called E*rag*, could be identified (Hsu *et al.*, 2003).

1.4.5. Rearrangement and expression of immunoglobulin genes

The recombination process itself is very similar for the heavy chain and both light chains. It differs only in the number of rearranged gene segments. While the light chain loci have only

V and J gene segments to be recombined, the V_H and J_H gene segments of the heavy chain are rearranged with an intermediate D_H element (summarized in Grawunder *et al.*, 1998).

Rearrangement starts at the heavy chain locus with the joining of one D_H segment to one J_H segment leading to the deletion of the intervening DNA. Subsequently a V_H gene segment rearranges to DJ_H. After generation of a functional heavy chain, the recombination of V_κ and J_κ at the κ locus takes place. A peculiarity of the κ locus is that some of the V_κ gene segments have an opposite transcriptional orientation to that of the J_κ gene segments. When the RSS in such cases are combined, the intervening DNA is not lost from the chromosome but is retained in an inverted orientation. Similar to the heavy chain locus the rearrangement of $V_\kappa J_\kappa$ does not always lead to a functional κ chain. In this case rearrangement at the second allele takes place. Only if the recombination on both κ alleles was not functional, the λ locus will be rearranged using the same rules described for the heavy or the κ light chain, respectively.

1.4.6. Proteins involved in V(D)J recombination

V(D)J recombination is initiated by Rag1 binding to the nonamer element of the RSS, followed by recruitment of Rag2 and interaction with the heptamer. Then, the Rag proteins introduce single-strand nicks precisely between the RSS and the coding segment. This is followed by Rag catalysis of a *trans*-esterification reaction in which the 3' OH of the coding strand invades the complementary DNA strand to form closed hairpin coding ends and blunt 5' phosphorylated signal ends (van Gent *et al.*, 1996). The four Rag-liberated DNA ends remain associated with Rag in a stable postcleavage synaptic complex (Fugmann *et al.*, 2000). Subsequently, the signal and coding ends are differentially processed and joined. The ends of the heptamer sequences are joined precisely in a head-to-head fashion to form the signal joint in a circular fragment of DNA, which is subsequently lost when the cell proliferates (Lewis *et al.*, 1985; Lieber *et al.*, 1988). The joining of the V, (D) and J gene segments, the so-called coding joint, is not precisely defined, and consequently generates extensive variability at the CDR3 of a heavy chain (see 1.4.7.). The joining phase of the V(D)J recombination reaction is carried out primarily by ubiquitously expressed non-homologous DNA end-joining (NHEJ) proteins, although the Rag proteins appear to be important for the joining phase of the reaction in the context of the postcleavage synaptic complex (Yarnell *et al.*, 2001). These NHEJ proteins are usually associated with general DNA double-strand break repair - like in the case of radiation-damaged DNA. Three such NHEJ proteins are subunits of the DNA-dependent protein kinase (DNA-PK), which consists of the Ku70 and Ku80 DNA-binding

subunits and a large catalytic subunit (DNA-PKcs) related to PI-3 kinases (Khanna and Jackson, 2001). Activation of DNA-PKcs requires binding of the Ku70/Ku80 complex to double-strand breaks. The role of DNA-PKcs first became apparent in the ionization radiation sensitive scid (severe combined immunodeficiency) mice, which lack B and T cell development due to a block of V(D)J recombination by a mutation of DNA-PKcs (Bosma and Carroll, 1991). Targeted disruption of this protein kinase leads to defects very similar to those caused by the naturally occurring scid mutation (Gao *et al.*, 1998a; Taccioli *et al.*, 1998). Similarly, defects in the Ku proteins interfere with V(D)J recombination (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gu *et al.*, 1997; Ouyang *et al.*, 1997). However, while DNA-PKcs-deficient cells are severely impaired only for coding joining, Ku-deficient cells are severely impaired in both coding and signal joining. The precise role of DNA-PKcs is still unknown, as its *in vivo* substrates are not identified till now. However, its function in NHEJ was suggested to involve end-processing, including hairpin opening, as indicated by the unusually large number of P nucleotides in DNA-PKcs-deficient cells (Lewis, 1994). In addition, *in vitro* studies have demonstrated that DNA-PKcs forms a complex with Artemis and phosphorylates it (Moshous *et al.*, 2001). This leads to the activation of an endonuclease activity that can cleave Rag-generated hairpins (Ma *et al.*, 2002). The analysis of Artemis-deficient mice demonstrated that this endonuclease activity is necessary to cleave coding end hairpins (Rooney *et al.*, 2002) and might also contribute to the loss of nucleotides in coding joints (Ma *et al.*, 2002; Schlissel, 2002b).

Two other important factors for V(D)J recombination are XRCC4, isolated on the basis of its absolute requirement for NHEJ and V(D)J recombination (Li *et al.*, 1995), and DNA ligase IV, implicated by its ability to form a complex with XRCC4 (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). Mice with one of these genes disrupted die late in fetal development due to primary defects in the nervous system (Gao *et al.*, 1998b; Frank *et al.*, 1998; Barnes *et al.*, 1998). However, transient transfection assays with targeted ES cells (Gao *et al.*, 1998b) or embryonic fibroblasts (Frank *et al.*, 1998) demonstrated the requirement of XRCC4 and DNA ligase IV for V(D)J recombination.

1.4.7. Generation of junctional diversity

Diversity of the antibody repertoire is achieved by several means: first by the random combination of separate V, (D) and J gene segments to a complete V region and second by the association of different heavy and light chain V regions to form the antigen-binding site.

Further diversification is due to junctional diversity. This is caused by the addition or deletion of nucleotides at the ends of coding sequences (Fig. 1-8).

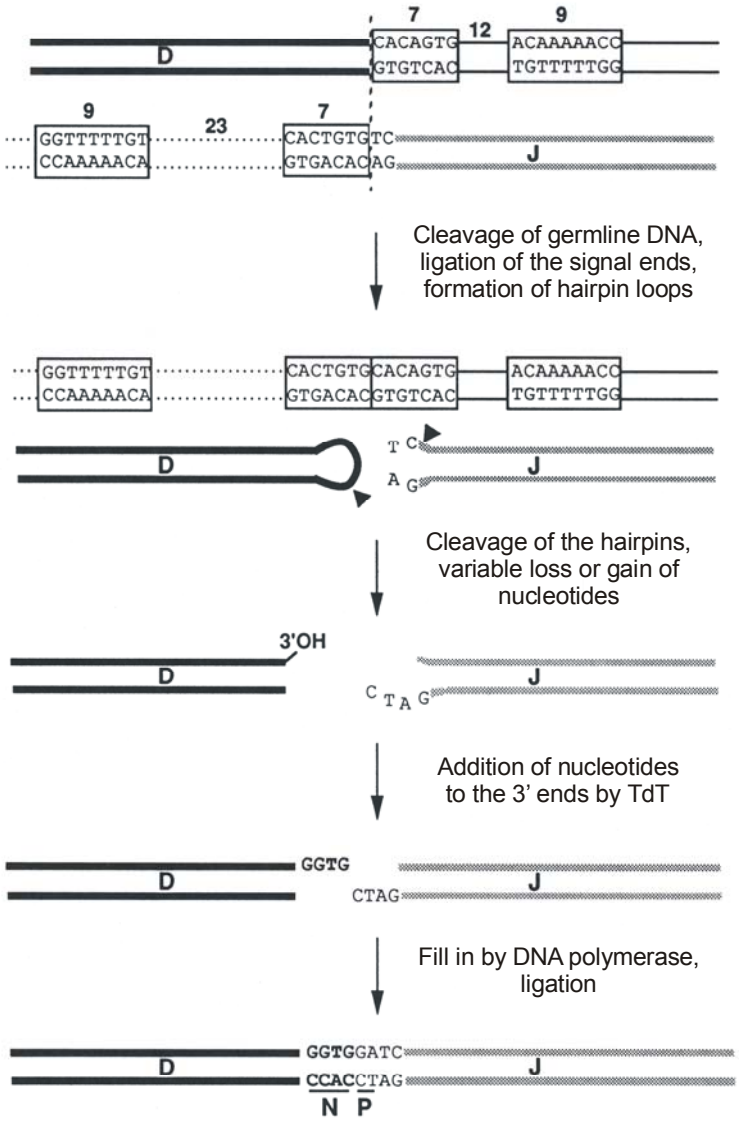


Fig. 1-8: Generation of junctional diversity. Schematically shown is the joining of a D and a J gene segment. Loss and gain of nucleotides can happen between every joined gene segment.

The coding end hairpins generated during the V(D)J recombination process are often opened asymmetrically. This activity has been proposed to be carried out by the Rag proteins and/or the nuclease Mre11 (Fugmann *et al.*, 2000; Paull, 2001), although the Artemis protein described before appears to be the prime physiological candidate now (Ma *et al.*, 2002). The asymmetric opening of the hairpin results in a single-stranded tail consisting of a few

nucleotides of the coding sequence plus the complementary nucleotides from the opposite DNA strand. DNA repair enzymes fill up the missing complementary nucleotides to complete the double strands again. Subsequent joining of the newly generated coding ends leads to short palindromic sequences at the joint, called P nucleotides (Lewis, 1994; Lafaille *et al.*, 1989; McCormack *et al.*, 1989).

The single-stranded tail can also be a target for exonucleases whose activity leads to a loss of nucleotides (Kenter and Tredup, 1991), a process called "nibbling". In addition endonuclease action inside the coding end has also been suggested for this activity (Lewis *et al.*, 1985; Besmer *et al.*, 1998). Although several exonucleases are known in mammalian cells, the specific enzyme responsible for the nibbling has not been definitely identified.

A third modification is the insertion of non-templated nucleotides, the N nucleotides, into the coding joints. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyses the addition of nucleotides to the 3' ends of the coding ends (Landau *et al.*, 1987). After the addition of up to 20 nucleotides, the two single-stranded stretches form base pairs over a short region. Repair enzymes trim off any non-matching bases, synthesize complementary bases to fill in the remaining single-stranded DNA and ligate them, resulting in the formation of an intact coding joint.

Mice with a targeted disruption of the TdT gene clearly demonstrate that TdT is responsible for the N nucleotide insertions. They have virtually no N nucleotides (Gilfillan *et al.*, 1993; Komori *et al.*, 1993). In contrast, TdT transgenic mice have increased numbers of N nucleotide insertions (Landau *et al.*, 1987; Bentolila *et al.*, 1997; Benedict and Kearney, 1999).

Two different splice variants of the TdT enzyme exist differing by a 60 bp insertion (Lo *et al.*, 1991; Doyen *et al.*, 1993). Both the short isoform (TdTS) and the long isoform (TdTL) of TdT were shown to mediate modifications of the coding joints in the V(D)J recombination process (Thai *et al.*, 2002; Benedict *et al.*, 2001). TdTS could be demonstrated to catalyze N nucleotide additions, while TdTL was found to have 3'→5' exonuclease activity on single-stranded DNA (Thai *et al.*, 2002).

The random number of inserted or deleted nucleotides by the generation of junctional diversity, leads very often to a frame-shift in the reading frame. Abortion of the translation due to stop-codons downstream is the result (summarized in Lewis, 1994). However, if the rearrangement on one allele is not functional, recombination at the second allele takes place. In the case of a functional joining the cell can be rescued from apoptosis as described before.

1.5. Regulatory elements of the transcription

The rearrangement of the immunoglobulin genes generates not only the variable regions of both the heavy and the light chain. The recombination process also brings regulatory *cis* elements, like promoters and enhancers, into functional proximity. Transcription of the Ig gene loci is regulated on several levels. On the one hand, interactions between *cis*-acting elements like promoters and enhancers and *trans*-activating, DNA-binding factors take place, influencing the capacity of the RNA polymerase II to initiate the RNA synthesis. Furthermore the chromatin structure, the nuclear matrix and other *cis*-acting elements like matrix attachment regions (MAR), locus control regions and silencers play a role (summarized in Max, 1999).

1.5.1. Enhancers of the immunoglobulin gene loci

A strong transcriptional enhancer of the heavy chain locus is localized in the large intron between the J_H gene segments and the C_μ gene (E_μ; Fig. 1-2). Targeted elimination of this enhancer led to an impairment of the V(D)J recombination of the corresponding allele (Serwe and Sablitzky, 1993; Chen *et al.*, 1993b). An additional enhancer was found about 16 kb 3' of the C_α region (3'E_H; Fig. 1-2). This enhancer has no influence on the recombination process. However, it plays a role for class switch recombination in later stages of B cell development (Cogne *et al.*, 1994).

For the κ locus two enhancers are known. One of them is located in the large intron between the J_κ gene segments and the C_κ segment (iE_κ), the other is positioned 9 kb 3' of the C_κ segment (3'E_κ; Fig. 1-4). *In vitro* studies using cell lines have shown that iE_κ is necessary for the demethylation and germline transcription of the κ light chain locus (Lichtenstein *et al.*, 1994; Klug *et al.*, 1994). Experiments with embryonic stem cells, in which iE_κ was replaced by a neomycin resistance cassette or by a recombinant *loxP* site, resulted only in an impaired rearrangement at the corresponding locus but not in a total block (Xu *et al.*, 1996).

The intron enhancer iE_κ has a binding site for the transcription factor NF-κB. This factor plays a key role in many immunological activation processes and stress responses (Lenardo and Baltimore, 1989). Mutations of the κB motif strongly reduced the enhancer activity pointing at a critical role of this enhancer function (Lenardo *et al.*, 1987).

The second enhancer, 3'E_κ, displays a sevenfold increased enhancer activity compared to iE_κ. It was demonstrated, that this enhancer alone is sufficient for the transcription of immunoglobulin genes in B cells (Meyer *et al.*, 1990). Deletion of 3'E_κ led to a reduction of κ expressing B lymphocytes (Gorman *et al.*, 1996).

In the λ light chain locus two enhancers have been identified till now. The E_λ2-4 enhancer is located 15.5 kb 3' of the C_λ4 gene segment, whereas the E_λ1-3 enhancer is positioned 35 kb 3' of the C_λ1 gene segment (Fig. 1-6; Hagman *et al.*, 1990). Both enhancers display binding motifs for transcription factors found also in the κ enhancer 3'E_κ, whereas a binding motif for NF-κB was not found (summarized in Max, 1999).

1.5.2. Promoters of the immunoglobulin gene loci

Each V gene segment of the heavy and the light chain gene loci has on its 5' site a promoter, which ensures the transcription of the rearranged chain. Before rearrangement already weak transcription is mediated by these promoters (Fig. 1-2; Fig. 1-4 and Fig. 1-6). Due to the recombination they are placed into functional proximity to the enhancers resulting in enhanced transcription.

Additional promoters were described mediating transcription of still unrecombined JC clusters. The gene locus is hereby transcribed in its germline configuration, therefore the promoters are named germline promoters and the transcripts are called germline transcripts respectively. Presumably, these germline transcripts do not encode proteins because of many start and stop codons within the transcripts. Similarly promoters driving transcription of rearranged DJ elements at the heavy chain locus are known.

Two promoters 5' of the first J_κ gene segment were described for the κ locus (Fig. 1-4; Van Ness *et al.*, 1981; Martin and Van Ness, 1990). At the λ locus, promoter activity for a region upstream of J_λ2 has been demonstrated (Engel *et al.*, 2001). In case of the heavy chain, the intron enhancer E_μ displays additional promoter activity. A sequence motif, called octamer motif, of this region plays a role that is more important for the promoter than for enhancer activity (Su and Kadesch, 1990). This promoter lacks a TATA box, important for a precise initiation point of the transcription (Su and Kadesch, 1990). Besides, additional promoters 5' of some heavy chain D gene segments were described (Reth and Alt, 1984). A bifunctional *cis* element, which displays both promoter and enhancer activity similar to Eμ, was identified for the most 3' D segment, D_{Q52} (Kottmann *et al.*, 1992; Kottmann *et al.*, 1994).

1.6. Regulation of V(D)J recombination

Rearrangement of receptor gene segments takes place only in well-defined stages of B and T cell development (Schatz *et al.*, 1992). Although both cell types use the same recombination machinery, B lymphocytes recombine exclusively immunoglobulin genes, while T lymphocytes rearrange only gene segments of the T cell receptor to completion (Willerford *et al.*, 1996). Only sporadically DJ recombination at the immunoglobulin gene locus was found in T cells, and although for both B and T lymphocytes two alleles exist for each locus, only one functional antigen receptor is generated (Rajewsky, 1996). This enormous specificity together with the fact that only a single recombinase is needed led to the suggestion that the accessibility of the gene loci and of the corresponding gene segments, respectively, must be strictly regulated for the recombination machinery (Yancopoulos and Alt, 1985; Yancopoulos *et al.*, 1986; Alt *et al.*, 1987). It was assumed therefore that the gene loci are first not accessible and become accessible to the recombination machinery only after some kind of modification. Changes in chromatin structure, demethylation of DNA and germline transcription might play a role because they were found to correlate with the appearance of recombinase activity.

It is accepted that DNA methylation influences the chromatin structure and leads to changes in gene expression (Ng and Bird, 1999). For both, the heavy chain and the κ light chain, it was demonstrated that early in B cell ontogeny the loci are hypermethylated (Mather and Perry, 1983; Storb and Arp, 1983). Parallel to induction of recombinase activity, these loci become hypomethylated and transcriptional active (Kelley *et al.*, 1988; Goodhardt *et al.*, 1993). In an *in vitro* experiment using B cell lines, demethylation of the κ locus alone did not lead to activation of the rearrangement, suggesting that demethylation is not the cause of changes in accessibility. However, in one of such cell lines germline transcription was induced (Cherry *et al.*, 2000).

More recent studies point out that the decisive step that makes the gene locus accessible to the recombination machinery, are changes of the nucleosomal structure (Kwon *et al.*, 1998; Cherry and Baltimore, 1999; Golding *et al.*, 1999). In this event the histone acetylation might play an important role. Several groups could demonstrate that histone acetylation stimulates V(D)J recombination (McMurry and Krangel, 2000; McBlane and Boyes, 2000; Kwon *et al.*, 2000). Histone acetylation is also involved in the transcriptional control of genes, e.g. the lymphoid transcription of the murine CD21 gene is positively regulated by histone acetylation (Zabel *et al.*, 1999).

1.6.1. The role of germline transcription for the recombination process

Weak transcription of the gene segments of the still unrearranged gene locus is a sign for the activation of the particular locus for the recombination process. This germline transcription shows probably the accessibility of the corresponding gene locus to the recombination machinery, because for both, the heavy chain locus (Yancopoulos and Alt, 1985) and the κ light chain locus (Schlissel and Baltimore, 1989; Lennon and Perry, 1990), a correlation between germline transcription and rearrangement was found. Similar results have been found for the gene loci coding for the T cell receptor (Fondell and Marcu, 1992; Goldman *et al.*, 1993; Holman *et al.*, 1993). Equivalent findings exist also for the human κ locus (Martin *et al.*, 1991).

Changes in the chromatin structure seem to result in accessibility of the gene locus to the recombination machinery. The question is whether these changes are the outcome of the germline transcription or, if the chromatin structure is influenced by other factors, germline transcripts are just an indicator for the altered accessibility. Up to now this question has not been answered. However germline transcription seems to play an important role in the recombination process as was shown by targeted deletion of regulatory elements essential for this transcription (see above).

1.6.2. Germline transcripts

Germline transcripts of the variable gene segments were found for both the heavy and the light chains (Schlissel and Baltimore, 1989; Blackwell *et al.*, 1986; Picard and Schaffner, 1984; Picard and Schaffner, 1985; Yancopoulos and Alt, 1985). For the μ heavy chain and both light chains transcripts are also known, which include the C gene segments (Alt *et al.*, 1982; Kemp *et al.*, 1980; Nelson *et al.*, 1983; Nelson *et al.*, 1984; Nelson *et al.*, 1985; Engel *et al.*, 1999). Two alternative transcripts were described for the heavy chain locus. One transcript initiates in the E_μ enhancer and contains a non-translatable exon, which is spliced to the C_μ region ($I\mu^0$; Lennon and Perry, 1985). This non-translatable exon is located in the intron between the J gene segments and the C_μ gene. Normally this exon will be deleted by splicing in the primary RNA transcript of a rearranged heavy chain. A second transcript initiates 5' of the D segment D_{Q52} and is, in its processed form, also spliced to the C_μ region (μ^0; Alessandrini and Desiderio, 1991).

For the κ light chain locus two transcripts have been described, which include the C_κ region. One germline transcript starts 3.5 kb 5' of the first J segment and leads to a primary transcript 8.4 kb in length (Perry *et al.*, 1980; Van Ness *et al.*, 1981), resulting in a 1.1 kb processed transcript (Martin and Van Ness, 1990). The second germline transcript initiates 50 to 100 bp 5' of the J segments (Leclercq *et al.*, 1989; Martin and Van Ness, 1990) and leads to a 0.8 kb processed transcript (Martin and Van Ness, 1990).

For the three functional $J_\lambda C_\lambda$ clusters of the λ locus germline transcripts were reported. They initiate 5' of the J segments and are spliced to the respective C region. Splice products of 0.6 kb in length are the result (Engel *et al.*, 1999; Engel *et al.*, 2001).

1.6.3. Role of germline transcription *in vivo*

Initially it was demonstrated for the α locus of the TCR, that deletion of the T early alpha (TEA) transcript, starting 5' of the J segments, resulted in altered J gene usage. J segments of the 5' part of the J_α cluster were no longer used in rearrangements, while J segments of the 3' part were utilized instead. This led to the suggestion, that additional transcripts could exist besides transcripts of the rearranged locus (Villey *et al.*, 1996). Subsequently a transcript of the β locus of the TCR was found, that initiates 5' of the $D_\beta 1$ segment. It is comparable to the μ^0 transcript of the immunoglobulin heavy chain locus. Deletion of the corresponding promoter prevents the germline transcription and results in an impaired rearrangement of the $D_\beta 1$ segment. In the first place, this is caused by diminished cleavage of the RSS through the Rag proteins (Whitehurst *et al.*, 1999).

For the κ light chain locus it has been shown, that deletion of a 4 kb fragment located 5' of the first J gene segment led to an impaired rearrangement both *in vitro* and *in vivo* (Cocea *et al.*, 1999). This fragment includes the promoter for the 1.1 kb germline transcript.

Due to several stop codons in the transcript, as mentioned above, most likely no translation product derived from such transcripts might exist. However, the germline transcript itself could play a role for the recombination process. In case of class switch recombination at the immunoglobulin heavy chain locus, transcription of the corresponding C segments could be observed similar to germline transcription preceding rearrangement. Studies at the IgG1 locus have shown, that modifications of the locus, preventing the processing of the germline or switch transcript, led to a block in the class switch recombination. In this case it was postulated that the switch transcript itself is directly involved in the class switch

recombination (Hein *et al.*, 1998). *In vitro* experiments have now shown, that the switch transcript forms a RNA-DNA hybrid with the template strand. This results in the exposure of a region of single-stranded DNA on the non-template strand that may be as long as 1 kb (Shinkura *et al.*, 2003; Yu *et al.*, 2003). This structure is called an R loop and presumably makes the switch region (S) accessible to activation-induced cytidine deaminase (AID) (see 1.8.). A similar role for the rearrangement could be played by the germline transcripts.

1.7. Cellular stages of the B cell development

B lymphocytes are generated from pluripotent, self-renewing hematopoietic stem cells (HSC) by a complex differentiation process, in liver during mid-to-late fetal development and in the bone marrow after birth. Such HSCs are Thy-1.1^{lo}Sca-1^{hi}c-kit^{hi} and do not express surface markers characteristic for different blood cell lineages. In the appropriate environment, these lineage marker (CD3, CD4, CD8, CD45R/B220, CD11b/Mac-1, Ly-6G/Gr-1, Ter119) negative cells (Lin^{-}) can give rise to cells of the erythroid, megakaryocytic, myeloid and lymphoid lineage (summarized in Morrison *et al.*, 1995). An early step in the differentiation process is the division into an interleukin-7 receptor α chain negative (IL-7Rα)$^{-}$ common myeloid progenitor (CMP; Akashi *et al.*, 2000b), which is able to generate all myeloid cell types (erythrocytes, megakaryocytes, granulocytes and macrophages), and into an IL-7Rα^{+} common lymphoid progenitor (CLP; Kondo *et al.*, 1997), that can give rise to all lymphocytes (B, T and natural killer (NK) cells). This model of adult hematopoiesis with the early separation between the myeloid and the lymphoid lineage although widely accepted is challenged by observations of progenitors with macrophage-B, macrophage-T or B-T potential (Montecino-Rodriguez *et al.*, 2001; Allman *et al.*, 2003).

1.7.1. Commitment of hematopoietic stem cells to the B lineage

Immediately following the CLP stage, a restriction of some cells to the B lineage can be observed which is characterized by the expression of CD45R/B220 (Allman *et al.*, 1999; Li *et al.*, 1996; Ogawa *et al.*, 2000). This molecule is also expressed on some non-B lineage cell types (including a subpopulation of NK cells) in the murine bone marrow, but in the context with other cell markers, it is possible to employ B220 to determine the earliest known progenitor cells committed to the B lineage. These pre-pro-B cells are B220^{+}CD43^{+}CD24/HSA^{lo} (Hardy *et al.*, 1991). They do not yet express the B cell marker

CD19, while all later stages in B cell development do (Li *et al.*, 1996; Rolink *et al.*, 1996). B cell progenitors of this fraction also express the complement component C1q like receptor C1qRp, a transmembrane protein that is recognized by the monoclonal antibodies 493 and AA4.1 (Petrenko *et al.*, 1999; Norsworthy *et al.*, 1999; see also Rolink *et al.*, 2002). In addition, commitment to the B lineage is characterized by the expression of genes that are involved in assembly and expression of the BCR complex. Thus, the signal transducing molecules Igα and Igβ and genes encoding the surrogate light (SL) chain, like VpreB1, VpreB2 and λ5, are expressed (Li *et al.*, 1996). Furthermore, germline transcripts of the heavy chain locus (μ^0) can be detected (Allman *et al.*, 1999).

The commitment to the B lineage appears to be mediated by the activity of several transcription factors (summarized in Schebesta *et al.*, 2002a; Johnson and Calame, 2003). One particular important role plays the PU.1 (Spi-1) protein, a member of the ets family of transcription factors. The analysis of PU.1 knockout mice demonstrated the critical role that PU.1 is playing in B cell development. Mice lacking PU.1 fail to generate mature myeloid or B lineage cells and accumulate variable numbers of T cells (Scott *et al.*, 1994; McKercher *et al.*, 1996). Elegant complementation experiments in which different levels of PU.1 were expressed using retroviral mediated gene transfer showed that high levels of PU.1 resulted in the differentiation of precursors into myeloid lineage cells, lower levels favor generation of B lineage cells (DeKoter and Singh, 2000). Partial restoration of B cell development following ectopic expression of IL-7Rα in PU.$1^{-/-}$ progenitors identifies IL-7Rα as a critical B cell target of PU.1 (DeKoter *et al.*, 2002). At the molecular level, low PU.1 concentrations activate the IL7-Rα gene, in contrast to high PU.1 levels, which prevent IL7-Rα expression (DeKoter *et al.*, 2002).

The commitment of a progenitor to the B lineage not only needs activation of a B cell specific program but also requires inhibition of programs driving alternative cell fates. A B cell specific member of the Pax family, Pax5, acts as a transcriptional activator on targets known to be critical in establishing B cell identity, including *Cd19*, *mb-1* (Igα) and *Blnk/SLP-65* (Nutt *et al.*, 1998; Schebesta *et al.*, 2002b). In addition, Pax5 is also required to repress alternative cell fates. It has been shown that $Pax^{-/-}$ $B220^{+}$ 'pro-B cells' of the bone marrow can differentiate into T cells, macrophages, dendritic cells, NK cells and even osteoclasts (Rolink *et al.*, 1999a; Nutt *et al.*, 1999a). Furthermore it was demonstrated that Pax5 determines the B versus T cell fate by repression of *Notch1*, which is critical for T lineage commitment and development, without influencing myeloid and NK cell ontogeny (Souabni *et al.*, 2002; Cotta

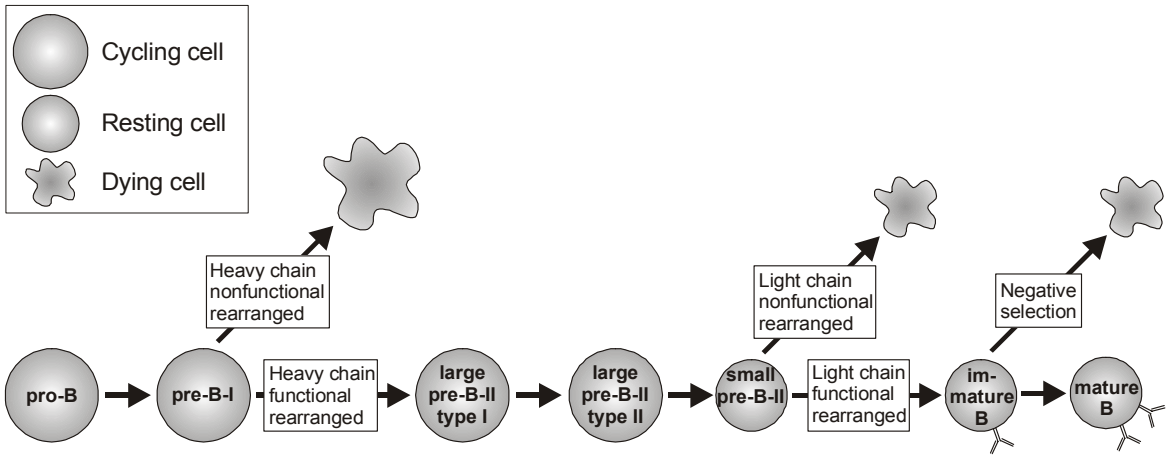
et al., 2003). Pax5 is induced in response to IL-7 (DeKoter *et al.*, 2002) and there is evidence that the transcription factors E2A and EBF control Pax5 expression (Greenbaum and Zhuang, 2002). Thus B cell commitment is established by a synergy and antagonism of regulatory mechanisms.

1.7.2. B cell development in the bone marrow

Using cell surface molecules and intracellular markers, different developmental stages can be distinguished during the development from B cell precursors to mature B lymphocytes in the bone marrow. Two nomenclatures are commonly used (Hardy *et al.*, 1991; Rolink *et al.*, 1999b). The system employed by Rolink and Melchers (Rolink *et al.*, 1999b) includes besides particular markers the rearrangement status of heavy and light chains and in addition the cell size. It also implicates that large cells are dividing cells whereas small cells are resting cells. This nomenclature will be used in the present work (Fig. 1-9).

The earliest B cell precursors in this system are the large (progenitor) pro-B and the (precursor) pre-B-I cells. They are characterized by the expression of B220 and CD19, cell surface markers found also on all later B cell stages. Plasma cells, which do not express B220, are the only exception. In addition, all B cell stages from the pro-B cells up to the immature B cells express C1qRp, which is recognized by the monoclonal antibody 493 (Rolink *et al.*, 1998).

Pro-B and pre-B-I cells express on their surface the receptor phosphotyrosine kinase c-kit (Ogawa *et al.*, 1991). Moreover, they are positive for the IL-7 receptor (Sudo *et al.*, 1993). Pro-B and pre-B-I cells differ in the status of rearrangement of the heavy chain, i.e. pro-B cells have the locus still completely in germline configuration while pre-B-I cells show already – often on both alleles – DJ_H recombination. Components of the recombination machinery like Rag1, Rag2 and TdT are detectable in these cells (Grawunder *et al.*, 1995a). In mice with a targeted inactivation of either the *Rag1* or *Rag2* gene and in scid mice, a complete block in the B cell development was observed at this stage indicating the requirement of rearrangement for further developmental progression (Melchers and Rolink, 1999).



		pro-B	pre-B-I	large pre-B-II type I	large pre-B-II type II	small pre-B-II	immature B	mature B
Ig gene loci	**H:**	N.R.	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
	L:	N.R.	N.R.	N.R.	N.R.	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$
Cell surface markers	**B220**	+	+	+	+	+	+	+
	CD19	+	+	+	+	+	+	+
	C1qRp	+	+	+	+	+	+	-
	c-kit	+	+	-	-	-	-	-
	IL-7R	+	+	+	+/-	-	-	-
	CD25	-	-	+	+	+	+/-	-
	CD40	-	-	-	-	+/-	+	+
	CD23	-	-	-	-	-	-	+
	sIgM	-	-	-	-	-	+	+
	sIgD	-	-	-	-	-	+/-	+
Intracellular markers	**Rag1**	-	+	-	+/-	+	+ (protein)	-
	Rag2	-	+	-	-	+	+ (protein)	-
	TdT	-	+	-	-	-	-	-
	$I\mu^0$	+	+	+	+	+	+	+
	SL	+	+	+	-	-	-	-
	cyto-μ	-	-	+	+	+	+	+
	κ^0	-	-	-	+	+	+	+
	κ/λ	-	-	-	-	-	+	+

Fig. 1-9: Cellular stages of B cell development in the bone marrow. The status of the Ig gene rearrangement and characteristic surface and intracellular markers are shown. H: heavy chain locus, L: light chain locus, N.R.: not rearranged, +: detectable, -: not detectable, protein: detectable only on protein level, SL: surrogate light chain, cyto-μ: cytoplasmic μ chain, $I\mu^0$: germline transcripts of the heavy chain locus, κ^0: germline transcripts of the κ light chain locus, λ^0: germline transcripts of the λ light chain locus.

Pro- and pre-B-I cells can be grown very efficiently in tissue culture on stromal cells in the presence of IL-7 or IL-3 (Rolink *et al.*, 1991; Winkler *et al.*, 1995). Removal of one of these components induces differentiation up to surface IgM positive ($sIgM^+$) immature B cells (Rolink *et al.*, 1991). This differentiation happens also in B cells from *Rag* deficient mice, although these mice can not rearrange their immunoglobulin genes, and leads to the activation of germline transcription at both light chain loci (Grawunder *et al.*, 1995b; Engel *et al.*, 1999). Due to the absence of rearrangement, these cells die within a few days, which could be prevented by the transgenic expression of the proto-oncogene *bcl-2* (Grawunder *et al.*, 1993; Rolink *et al.*, 1993a).

During the progression from pre-B-I cells to the next developmental stage, further rearrangement at the heavy chain locus takes place. Now a V_H gene segment is recombined with the DJ_H resulting in a complete V_HDJ_H transcription unit. Due to the generation of diversity at the coding ends (see above) the maintenance of reading frames is not guaranteed. Therefore, many recombination events end in non-functional V_HDJ_H recombinations. As a rule the rearrangement starts initially only at one allele. If no functional rearrangement is achieved, the recombination process proceeds at the alternative allele. Only a functional rearrangement results in progression of development into large pre-B-II cells. Although two heavy chain loci exist, the majority of the B lymphocytes only express one functional heavy chain. This phenomenon is called allelic exclusion. The molecular mechanisms for the regulation of allelic exclusion are still unclear (Schlissel, 2002a).

In large type I pre-B-II cells the expression of c-kit and TdT is downregulated. The α chain of the IL-2 receptor, the surface marker CD25, is expressed instead (Rolink *et al.*, 1994). Thus, CD25 serves as marker for the whole pre-B-II compartment. The functional V_HDJ_H transcription unit is expressed as cytoplasmic μ chain and is displayed at the cell surface in association with the SL chain and the Ig accessory proteins Igα and Igβ as pre-BCR. The SL chain consists of the two proteins λ5 and VpreB1 or VpreB2 (Martensson *et al.*, 2002). The importance of the presentation of the pre-BCR on the cell surface for the further developmental transition, has been shown in mice lacking VpreB1 (Martensson *et al.*, 1999), VpreB1 and VpreB2 (Mundt *et al.*, 2001), λ5 (Rolink *et al.*, 1993b; Kitamura *et al.*, 1992) or all components of the SL chain together (Shimizu *et al.*, 2002). All these mice exhibit a severe, although incomplete block in the development at the pre-B-I stage. Similar results were obtained from mice lacking the transmembrane portion of the μ heavy chain (μMT;

Kitamura *et al.*, 1991) or Igβ (Gong and Nussenzweig, 1996), which show an almost complete block after the DJ_H rearranged pre-B-I stage.

Subsequent to surface expression of the pre-BCR the members of the recombination machinery, Rag1 and Rag2, are rapidly downregulated (Grawunder *et al.*, 1995a). It was suggested that this should avoid VDJ recombination at the second allele, thus ensuring allelic exclusion. Although allelic exclusion takes place in pre-BCR bearing cells, the contribution of the pre-BCR itself is controversial (summarized in Martensson *et al.*, 2002). Direct evidence for the role of the pre-BCR in mediating heavy chain allelic exclusion has come from $\lambda5^{-/-}$ (Loffert *et al.*, 1996) and μMT mice (Kitamura and Rajewsky, 1992), which are 'allelic included' i.e. both heavy chain alleles are often functional rearranged. In contrast, another study describes the presence of a small fraction of pre-B-II cells with two functional rearranged heavy chain alleles in $\lambda5^{-/-}$ but also in normal mice (ten Boekel *et al.*, 1998). In these cases, only one μ heavy chain pairs with the SL chain whereas the other can not. Hence, allelic exclusion might be regulated by pairing properties of the μ heavy chain with the SL chain. This would suggest that the λ5 defect does not abolish allelic exclusion which was confirmed at the mature stage of B cells (ten Boekel *et al.*, 1998). However, it remains unclear which other complex if not the normal pre-BCR, composed of a μ heavy chain and λ5 and VpreB, could signal allelic exclusion, since the heavy chain locus in VpreB1, VpreB2 and λ5 triple deficient mice does not appear to be allelically 'included' (Shimizu *et al.*, 2002).

Large proliferating type II pre-B-II cells represent the developmental stage following large type I pre-B-II cells. Such cells have lost the expression of the SL chain and re-express *Rag1* and *Rag2* at the mRNA level (Grawunder *et al.*, 1995a). Although the light chain loci of these cells are still in germline configuration (ten Boekel *et al.*, 1995), germline transcripts of the κ light chain locus become detectable, a sign for the activation of the locus for recombination (Grawunder *et al.*, 1995b; Engel *et al.*, 1999).

Cells of the consecutive developmental stage, the small pre-B-II cells, have left the cell cycle. The IL-7 receptor is no longer expressed. The surface marker CD40 becomes detectable for the first time although a function of this molecule at this stage of development is not known (Rolink and Melchers, 1996). *Rag1* and *Rag2* expression becomes detectable now not only at the mRNA but also the protein level (Grawunder *et al.*, 1995a). In addition, germline transcripts of the λ locus appear (Engel *et al.*, 1999). A large fraction of such small pre-B-II cells have already rearranged light chain loci (ten Boekel *et al.*, 1995). Allelic exclusion exists also for the four light chain loci similar to the heavy chain locus. Furthermore isotype

exclusion takes place i.e. either the κ or the λ locus is functional rearranged, and functional light chain can be detected as cytoplasmic protein, but not yet on the cell surface (Engel *et al.*, 1998). Already at this stage of development the ratio of 10 : 1 between κ and λ expressing cells characteristic for the mouse could be observed (ten Boekel *et al.*, 1995).

This ratio of 10 : 1 between κ and λ expressing cells could be explained by a sequential activation of the light chain loci. The κ locus was shown to be activated for rearrangement in large pre-B-II cells, whereas λ activation became only detectable at the consecutive developmental stage of the small pre-B-II cells (Engel *et al.*, 1999). In this study, it was concluded that pre-B-II cells have only a limited life span during which they have to rearrange the light chains. Either they are rescued by the expression of a functional light chain or they die by apoptosis. Therefore the κ : λ ratio seems to be in part a result of the sequential activation of the light chain loci and the limited time available for rearrangement (Engel *et al.*, 1999).

Small pre-B-II cells that have undergone a productive light chain rearrangement can differentiate into $sIgM^+$ immature B cells. These cells express their rearranged heavy and light chains on the cell surface as BCR. The BCR is, similar to the pre-BCR, complexed with the signal transduction molecules Igα and Igβ. Mice with a targeted disruption of the κ intron enhancer iE_κ (Takeda *et al.*, 1993) or a deleted JC_κ region (Chen *et al.*, 1993a) show an impaired transition from the small pre-B-II cells to the immature compartment, displayed by drastically decreased numbers of immature B cells. Thus, the inability to produce a κ light chain protein greatly effects the transition from small pre-B-II cells to immature B cells. The low efficiency of λ light chain rearrangement (Ramsden and Wu, 1991) and the late activation of the λ locus for rearrangement together with the postulated limited life span appear to be a likely explanation for this defect.

Presumably, negative selection of auto-reactive B cells takes place at this developmental stage. It has been shown that *in vitro* stimulation of immature B cells with antibodies specific for sIgM induces apoptosis in these cells (Rolink *et al.*, 1998). *In vivo*, anti-hen egg lysozyme (HEL) specific immature B cells in mice transgenic for HEL show a developmental arrest in the bone marrow at this stage. These cells downregulate the expression of B220 and sIgM (Hartley *et al.*, 1993).

Auto-reactive B cells can be rescued from apoptosis by a process called receptor editing. Hereby, further rearrangements mainly at the light chain loci lead to the deletion of the anti-

self specificity, which otherwise would lead to apoptosis, and replacement by a new receptor. This enables the cell to complete the maturation process (summarized in Nussenzweig, 1998; Melchers *et al.*, 1995).

After surviving negative selection, the immature B cells leave the bone marrow and migrate into the spleen, where the final maturation takes place (Loder *et al.*, 1999). Intermediate stages in the spleen are defined as transitional 1 and 2 B cells and characterized by surface expression levels of CD21 and CD23 (Loder *et al.*, 1999).

Such developmental stages are well defined for bone marrow derived cells only. For fetal liver derived B lymphocytes the intermediate developmental stages and places of differentiation are less well defined.

1.8. Antigen-dependent diversification of immunoglobulins in mature B cells

The majority of the newly formed naïve B lymphocytes dies within the first 3 to 4 days, while only 5 to 10 % of them transit into the pool of relatively long-lived mature B cells (Forster and Rajewsky, 1990). These cells recirculate through the secondary lymphoid organs, like spleen, lymph nodes and Peyer's patches and have a life span of 3 to 8 weeks. If they encounter the antigen during this time via their BCR, the cells are activated, which normally also requires help from T cells. Three different processes affecting the structure of the Ig molecule can take place subsequent to activation. First, such activated B cells could differentiate after proliferative expansion into antibody secreting plasma cells (summarized in Baumgarth, 2000). The secretion of the formerly membrane-bound Ig molecule is achieved by alternative processing of the primary heavy chain transcript. The secreted form differs from the membrane-bound form by the addition of a short secretory domain and the absence of the C-terminal transmembrane domain and the short cytoplasmic.

The second diversification takes place during expansion phase. At the beginning of an antibody response IgM antibodies display a low affinity for antigen. Later, and within the scope of a secondary response after renewed antigen encounter, high-affinity antibodies are found. These antibodies are produced by B cell clones after so called affinity maturation. During this process mutations (mainly base substitutions, rarely deletions or insertions) are introduced into the V regions of the rearranged gene loci, commonly referred to as somatic hypermutation (summarized in Li *et al.*, 2004). Clones producing high-affinity antibodies are

selected and preferentially expanded during the germinal center reaction (Wolniak *et al.*, 2004). The mechanism underlying this process has recently elucidated and involves the activation-induced cytidine deaminase (AID) together with other DNA repair enzymes (Besmer *et al.*, 2004).

The third process affecting the Ig structure is the class switch recombination (CSR). In this reaction the C_H gene segment is replaced by another C segment ($C_{\gamma1}$, $C_{\gamma2a}$, $C_{\gamma2b}$, $C_{\gamma3}$, C_ε or C_α) changing the antibody class, whereas the antigen specificity of the B cell remains the same (Fig. 1-10). After the differentiation into plasma cells now the antibodies secreted by them possess a different effector function.

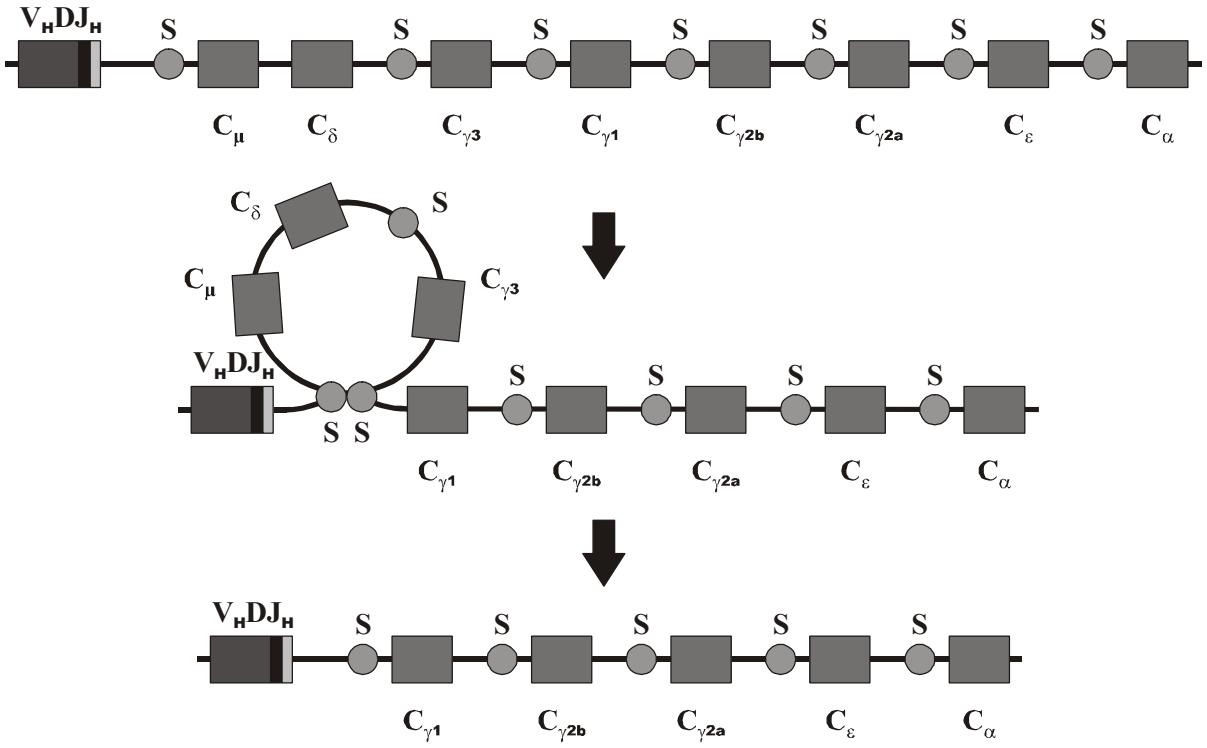


Fig. 1-10: Mechanism of class switch recombination. Shown is a V_HDJ_H rearranged heavy chain, where the switch from IgM to IgG1 takes place. The DNA between the recombined switch regions is excised, including the C_μ, C_δ and $C_{\gamma3}$ segments. The different C regions are displayed as single exons. S: switch region.

The CSR takes place by looping out and deleting DNA sequences located between two repetitive DNA sequences, the so called switch regions, that are located 5' of each C region except of C_δ. The selection of the C segment and consequently the isotype of the secreted

antibody are driven by cytokines, that are provided by T cells. Such regulation of CSR was shown by *in vitro* experiments. For instance, IL-4 causes the switch to γ_1 and ε, whereas interferon-γ leads to γ_{2a} and TGF-β to α (summarized in Snapper and Finkelmann, 1999).

AID that was shown to be crucial for somatic hypermutation is surprisingly also essential for CSR (Muramatsu *et al.*, 2000; Revy *et al.*, 2000). But it is still unclear, how AID links these two processes that did not appear to be mechanistically associated before (Longacre and Storb, 2000; Li *et al.*, 2004).

Finally, after antigen encounter, B cells could leave the cell cycle and differentiate into long-lived memory cells (Maruyama *et al.*, 2000). This process does not affect the Ig structure, but it enables the body to react faster in the case of renewed antigen i.e. pathogen encounter.

1.9. Mature B cell subpopulations and their functional properties

The above properties and mechanisms are mainly attributed to a B cell population denominated B2 cells. However, there are additional B cell populations or subpopulations. Based on cell surface markers and anatomical localization the mature B cell compartment can be divided into B1 and B2 cells that display different functional characteristics (Kantor *et al.*, 1992; Kantor *et al.*, 1995b). The B2 cell population itself is heterogeneous and can further be subdivided into follicular (FO) and marginal zone (MZ) B cells. FO B cells recirculate through the peripheral lymphoid organs and locate in the B cell follicles of spleen and lymph nodes. They represent the major splenic B cell population. FO B cells are characterized by the cell surface expression of intermediate levels of CD21 ($CD21^{int}$), low levels of IgM (IgM^{lo}), high levels of IgD (IgD^{hi}) and additional expression of CD19, B220 and CD23 ($CD19^{+}B220^{+}CD23^{+}$).

MZ B cells are defined as $CD19^{+}B220^{+}CD23^{lo/-}CD21^{hi}IgM^{hi}IgD^{lo}$ cells in the spleen, where they are located as a mostly non-recirculating population in the marginal zone (Gray *et al.*, 1982; Oliver *et al.*, 1997). The marginal zone is a distinct anatomical compartment that in mice is only found in the spleen and surrounds the B cell follicles and the periarteriolar T cell areas.

Originally, B1 cells were identified by their moderate expression of CD5 (Ly-1), a glycoprotein normally expressed on T cells. They are the predominating B cell population in peritoneal and pleural cavities. B1 cells can also be distinguished from B2 cells, which are found in such cavities as well, by the expression level of additional surface markers. They are

$CD19^{hi}B220^{lo}IgM^{hi}IgD^{lo}CD43^{+}$ and express the macrophage marker Mac-1 (CD11b/CD18). After the initial discovery of the CD5 expressing B1 cells, a population of peritoneal $CD5^{-}$ B cells was identified whose surface phenotype was in other respects identical to that of B1 cells. By consensus, peritoneal B1 cells were then subdivided into the $CD5^{+}$ B1a cells and the $CD5^{-}$ B1b "sisterpopulation".

B1a cells could also be found in the spleen, where they do not express Mac-1. Although they represent only a minor population of the splenic B cell compartment, their absolute number is similar to the number of B1a cells found in the peritoneum (Kantor *et al.*, 1992). Due to their low frequency, B1a cells from the spleen have not been studied to a large extent so far.

The phenotypically and spatially separated mature B cell subsets are also characterized by different functional properties. FO B cells participate in T cell dependent (TD) antibody responses (Martin and Kearney, 2000a). Hereby FO B cells are able to differentiate into antibody secreting plasma cells after antigen encounter and T cell help. Furthermore, they can increase the specificity of their receptors by somatic hypermutation and change their functional properties by switching their Ig class. FO B cells could also leave the cell cycle and differentiate into long-lived memory cells, which enables the body to react faster and more efficient in the case of a re-infection by the same pathogen.

MZ B cells contribute substantially to the T cell independent (TI) immune response, but they are also able to respond to TD antigens (Martin and Kearney, 2000a; Martin *et al.*, 2001). TI antigens can be classified into two types, which activate B cells by different mechanisms. Antigens of the first type, the TI-1 antigens, contain an intrinsic activity that can directly induce the proliferation of B cells. One example are the lipopolysaccharides of gram-negative bacteria. High concentrations of these molecules cause proliferation and differentiation of B cells, regardless of their antigen specificity. This is known as polyclonal activation. On the other hand, TI-2 antigens are molecules with highly repetitive structures, probably acting by extensively crosslinking the surface immunoglobulins of specific B cells. This is not sufficient to drive responding B cells into antibody production because additional signals supplied by non-cognate interactions with T cells, NK cells, and perhaps other cells are necessary (summarized in Vos *et al.*, 2000).

In TD immune responses MZ B cells capture, process and present antigen, and deliver co-stimulatory signals to T cells faster and more efficiently than FO B cells (Martin and Kearney, 2002). They have also the capacity to rapidly differentiate into plasma cells. Since FO B cells

participate also in TD immune responses, the main difference seems to be the time frame of involvement of MZ versus FO B cells (Martin and Kearney, 2002).

The current model for the function of MZ B cells is based on the specialized location near to the marginal sinus. Here they are exposed to blood-borne antigens, possibly carried by dendritic cells (Balazs *et al.*, 2002), and could interact with various other cell types of the MZ including dendritic cells and macrophages. It was hypothesized that MZ B cells, as excellent antigen-presenting cells, could participate, together with dendritic cells, in early stages of T cell activation (Martin and Kearney, 2002). The functional characteristics together with the anatomical localization have led to the suggestion that MZ B cells represent a "first line of defense" against blood-borne antigens (Martin and Kearney, 2000a; Martin and Kearney, 2002).

Since the initial discovery of ($CD5^+$) B1 cells 20 years ago, great efforts, especially in the past few years, have been carried out to clarify their origins and functions. Their precise role in the immune system however is still unclear, especially for the B1b subset (Herzenberg, 2000). Although, there are shared functional characteristics between B1a and MZ B cells, both populations exhibit also essential differences (Martin and Kearney, 2001; Kretschmer *et al.*, 2003a). Similar to the MZ B cells, they display a pre-activated phenotype. Both MZ and B1 cells are early participants in TI immune responses and give rise to the early wave of plasma cells in these responses (Martin *et al.*, 2001). B1a cells are known to have a limited capacity to switch to other Ig classes after antigen stimulation and therefore mainly express IgM (Tarlinton *et al.*, 1995). They are also believed to be the primary source of natural IgM. Natural IgM, which is produced in the absence of exogenous antigenic stimulation (Coutinho *et al.*, 1995; Casali and Schettino, 1996), is often poly-reactive, weakly auto-reactive, and reacts with many common pathogen-associated carbohydrate antigens. Due to its polymeric structure, secreted IgM has the ability to bind multimeric antigen and to efficiently activate the classical complement cascade. In addition, polymeric IgM can be transported via the poly-Ig receptor onto mucosal surfaces to provide protection from pathogenic invasion (Brandtzaeg, 1989; Gerhard *et al.*, 1997). These properties and the fact that IgM is the first class of Ig produced during an infection, have led to the suggestion that B1a cells also act as a "first line of defense" against mucosal and systemic pathogens. For instance, antibodies produced by B1a cells have been shown to play an important role in protection against infection by *Streptococcus pneumoniae* (Briles *et al.*, 1981; Yother *et al.*, 1982). A non-redundant role for B1a derived IgM has also been shown in the protective response to influenza virus infection (Baumgarth *et al.*, 2000). In addition, an important function of

natural antibodies for immune defense against certain pathogens has been demonstrated in mice deficient in antibody production by reconstitution with naïve serum (Ochsenbein *et al.*, 1999).

Still controversial is the contribution of B1a cells to the IgA producing cells in the gut. Transfer of total peritoneal B cells (Kroese *et al.*, 1989; Fagarasan *et al.*, 2000) or sorted B1 cells (Macpherson *et al.*, 2000) into recipients resulted in the appearance of donor-derived IgA plasma cells in the gut. After demonstration of *in situ* class switch in the lamina propria (Fagarasan *et al.*, 2001), this possibility has been suggested for B1 cells, since they are rarely found in germinal centers (Stall *et al.*, 1996), where the class switch in B cells normally takes place.

As B1 cells often produce auto-reactive antibodies, although of low affinity, it has been suggested that they might contribute to the anti-self antibody production in autoimmune diseases. Indeed, there are examples, in both human and mouse, of an association between autoimmune disease and B1 cells. In NZB/NZW mice cells with a B1 phenotype accumulate in the MZ prior to the appearance of clinical signs of systemic lupus erythematosus (SLE; Wither *et al.*, 2000). Nevertheless, B1 cells are not usually associated with autoimmune diseases. In MRL/lpr mice, another murine model of SLE, it was demonstrated that B2 and not B1 cells are necessary for disease pathogenesis (Reap *et al.*, 1993). A novel concept, therefore, implicates that these auto-reactive antibodies represent a distinct immune-recognition strategy, targeting conserved self-structures which are induced or exposed only in conditions of stress or tissue damage (Bendelac *et al.*, 2001).

The B1a and the MZ B cell compartments have been found to be enriched for certain B cell specificities (Herzenberg *et al.*, 2000; Martin and Kearney, 2000a; Kretschmer *et al.*, 2002). It has been suggested that this restriction together with the pre-activated phenotype should allow a rapid response against a limited number of conserved antigens. B1a together with MZ B cells are therefore part of a “natural memory” response and generate effectors rapidly in the first stages of an immune response. In contrast, the FO B cells provide a diverse pool of specificities from which B cells are recruited at later stages of the immune response, whereby high affinity memory is generated (Martin and Kearney, 2000a; Martin and Kearney, 2001; Martin and Kearney, 2002).

1.10. Origins of the mature B cell subpopulations

B lymphocytes are generated from HSCs in liver during mid-to-late fetal development and in the bone marrow (BM) after birth. The first appearance of the mature B cell subpopulations is consistent with these different anatomical locations and developmental periods. FO and MZ B cells are detectable for the first time after birth and are therefore the outcome of B cell development in the BM, whereas maturation into B1 cells takes place already in the fetal liver (FL; Akashi *et al.*, 2000a; Busslinger *et al.*, 2000). It was suggested that the generation of B1 cells is completed shortly after birth and their contribution to the mature B cell compartment throughout life is achieved by their capacity of self-renewal (Hayakawa *et al.*, 1985; Hayakawa *et al.*, 1986).

Reconstitution experiments gave rise to the proposition that B1 cells are developmentally distinct from the B2 cell population that is found in peripheral lymphoid organs, i.e. B1 and B2 cells derive from distinct progenitors. Adoptive transfers of embryonic splanchnopleura (Godin et al., 1993) or fetal omentum (Solvason et al., 1991) have shown that these sites contain progenitors exclusively for B1 cells and not for B2 cells. In contrast, fetal liver (day 13 and 14) contains progenitors for both of such cells (Kantor *et al.*, 1992; Solvason *et al.*, 1991). A proposed explanation for this co-existence of both progenitors is as follows: the fetal omentum is contiguous with the liver capsule. Therefore, as fetal omentum contains B1 but not B2 progenitors, it is possible that some or all of the B1 progenitors detected in fetal liver are located in the liver capsule or in co-isolated omental tissue. The B2 progenitors, in contrast, might be found in the fetal liver, but not in the omentum (Herzenberg, 2000).

Reconstitution with adult bone marrow demonstrated that progenitors for B2 cells are abundant at this site and readily reconstitute the full adult B2 population. In contrast, the adult bone marrow failed to reconstitute the B1 compartment (Kantor *et al.*, 1992; Kantor and Herzenberg, 1993; Kantor *et al.*, 1995b). A difference between B1a and B1b cells could be observed. While B1a cells were very poorly reconstituted from progenitors in adult bone marrow, B1b cells are sometimes reconstituted to half their normal frequency (Kantor *et al.*, 1992; Kantor *et al.*, 1995b). Since B1b typically represent 5 to 10 % of the overall B1 population, this B1b reconstitution is numerically not very significant (Herzenberg, 2000).

The functional existence of B1 progenitors independent of progenitors for B2 cells was taken as evidence for the existence of separate B cell lineages. Thus, the present data are most consistent with the idea that B1a and B2 cells (and perhaps also B1b cells) represent distinct developmental branches of the B lineage (Herzenberg, 2000). Further evidence for this

'lineage model' came from mice in which the neonatal B cells were depleted for 6 weeks by treatment with anti-IgM antibodies. The recovery of B2 cells begins as soon as the antibody treatment disappears and finally the B2 compartment reconstitutes completely. In contrast, B1 recovery fails entirely or is significantly disrupted, depending on whether the antibody treatment removed all B1 cells or only part of the (allotype-marked) B1 cells (Hamilton and Kearney, 1994; Lalor *et al.*, 1989a; Lalor *et al.*, 1989b).

However, there is also accumulating evidence that the differences between B1 and B2 cells are solely derived from differences in the specificity of their receptors (Cong *et al.*, 1991; Berland and Wortis, 2002). The basic premise of this 'induced differentiation model' is that B1 and B2 cells arise from the same progenitor but express Ig receptors whose antigen-binding specificity determines whether the newly formed B cells are triggered to differentiate into B1 or B2 cells. In this model, the fetal/neonatal repertoire is skewed toward the expression of immunoglobulins that bind frequently encountered TI-2 antigens resulting in the appearance of B1 cells. The adult repertoire rarely generates these specificities (due to, for instance, TdT expression) and therefore generates only few B1 cells (Berland and Wortis, 2002).

Evidence for this model came from the analysis of Ig heavy chain transgenic mouse models. Mice expressing a B1a derived BCR as transgene show an increase of the B1a population, while, conversely, in mice carrying B2 derived transgenes, the transgene expressing B cells are almost exclusively of the B2 phenotype (Table 1-1; summarized in Berland and Wortis, 2002).

Table 1-1: Effect of Ig transgene expression on B cell development (Berland and Wortis, 2002).

Transgene	Specificity	Source	Phenotype[a]	References
3-83	H-2$K^k D^k$ (MHC class I)	Adult spleen	B2	(Nemazee and Burki, 1989)
HyHEL-10	Hen egg lysozyme	Adult spleen	B2	(Goodnow *et al.*, 1988)
V_H81X	Unknown	Fetal liver	MZ	(Martin and Kearney, 2000b)
M167	Phosphoryl choline	Adult spleen	MZ	(Martin and Kearney, 2000b)
$V_H12/V_\kappa4$	Phosphatidyl choline	$CD5^+$ lymphoma	B1a	(Arnold *et al.*, 1994)
$V_H11/V_\kappa9$	Phosphatidyl choline	B1 cell	B1a	(Chumley *et al.*, 2000)
SM6C10	Thy-1	B1 cell	B1a	(Hayakawa *et al.*, 1999; Hayakawa *et al.*, 2003)
4C8	Mouse red blood cells	NZB spleen	B1	(Okamoto *et al.*, 1992)
2-12H	sM snRNP	MRL/lpr mouse	B1a	(Qian *et al.*, 2001)

[a] Predominant phenotype of B cells expressing the transgene

Similarly, the receptor specificity may play also a role in the decision between FO and MZ B cells. The light chain repertoire in heavy chain transgenic mice that selects B cells into the MZ compartment was found to be highly restricted (Martin and Kearney, 2000b). Although these data can be interpreted in the way that the receptor specificity is sufficient to determine the phenotype, so far it has not been shown that the B1 cells in the mice described above originated from adult BM rather than from fetal sources.

Table 1-2: Genetic alterations that affect B1 cell numbers (Berland and Wortis, 2002).

Alterations that decrease B1 cell numbers	**References**
Mutation of positive regulators of BCR signaling:	
Btk$^{-/-}$ or point mutation (xid) of btk	(Khan *et al.*, 1995; Hendriks *et al.*, 1996)
PKCβ$^{-/-}$	(Leitges *et al.*, 1996)
PLCγ$^{-/-}$	(Wang *et al.*, 2000; Hashimoto *et al.*, 2000)
Deletion of P85α of PI-3 kinase	(Fruman *et al.*, 1999; Suzuki *et al.*, 1999)
CD19$^{-/-}$	(Engel *et al.*, 1995; Rickert *et al.*, 1995)
SLP65$^{-/-}$	(Pappu *et al.*, 1999; Jumaa *et al.*, 1999)
CD21/35$^{-/-}$	(Ahearn *et al.*, 1996; but see Molina *et al.*, 1996)
Vav-1$^{-/-}$, Vav-2$^{-/-}$ and Vav-1$^{-/-}$/Vav-2$^{-/-}$	(Tarakhovsky *et al.*, 1995; Doody *et al.*, 2001; Tedford *et al.*, 2001)
Mutation of B cell transcription factors:	
Oct 2$^{-/-}$	(Humbert and Corcoran, 1997)
Aiolos$^{-/-}$	(Wang *et al.*, 1998)
NFATc$^{-/-}$	(Berland and Wortis, 2002)
Alterations that increase B1 cell numbers	
Mutation of negative regulators of BCR signaling:	
Loss of function of SHP-1 (*motheaten*, *motheaten*^{v})	(Sidman *et al.*, 1986)
SHP-1$^{-/-}$	(Schmidt *et al.*, 1998)
CD22$^{-/-}$	(Sato *et al.*, 1996a; O'Keefe *et al.*, 1996; but see Nitschke *et al.*, 1997; Otipoby *et al.*, 1996)
Lyn$^{-/-}$	(Chan *et al.*, 1997)
CD72$^{-/-}$	(Pan *et al.*, 1999)
Overexpression of positive regulators of BCR signaling:	
CD19 transgenic	(Sato *et al.*, 1996b)

Thus, the phenotypes of the transgenic mice where B1 cells are enriched could be explained by different selection processes taking place in B1 and B2 progenitors (Herzenberg, 2000). B1 cells in such mice could be derived from FL like in normal mice and the adult bone marrow might contribute only little to B cell ontogeny. Further support for different selection processes came from SM6C10 mice (Table 1-1), that express a heavy chain transgene derived from a naturally occurring auto-antibody specificity. In these mice, it has been shown that the presence of the particular B1a specificity depends on the interaction with the self-antigen (Hayakawa *et al.*, 1999), whereas B cells with such auto-reactive specificity are negatively selected during adult BM development (Hayakawa *et al.*, 2003).

The critical influence of BCR receptor signaling for B1 cell development was shown in different gene targeted and transgenic mice (summarized in Berland and Wortis, 2002). Mutations that disrupt or attenuate BCR receptor signaling, result in a substantial loss of the B1 cell subpopulation. On the other hand, inactivation of negative regulators of the BCR receptor complex or transgenes that enhance BCR signaling result in an expanded B1 compartment (Table 1-2). Consistently, in transgenic mice in which B cells display a high BCR receptor density, mainly B1 cells are found (Engel *et al.*, 1998; Lam and Rajewsky, 1999; Watanabe *et al.*, 1999).

1.11. Differences in lymphopoiesis between fetal liver and bone marrow

Comparison of lymphopoiesis in fetal liver and bone marrow reveals many similarities between both processes. The above described basic concept of B cell development, with its correlation between cell surface and intracellular markers and the status of Ig gene rearrangement, is applicable to both hematopoietic sites. However, many differences in lymphopoiesis between fetal and adult life have been described (Douagi *et al.*, 2002; Kincade *et al.*, 2002; Hardy, 2003).

An early step in the BM differentiation process is the division into CLP and CMP. An equivalent population to the CLP with regard to cell markers was found in FL. However, this precursor still has the capacity to generate dendritic cells and macrophages, in addition to T, B and NK cells (Mebius *et al.*, 2001). Also, a tripotent precursor, which gives rise to T cells, B cells and macrophages, has been described (Lacaud *et al.*, 1998). Like in the bone marrow, a rare population was isolated from fetal liver which has the ability to differentiate into B cells

and macrophages but not into other myeloid cells (Cumano *et al.*, 1992). Further evidence for a link between lymphoid and myeloid development came from the finding of bi-potential precursors giving rise to either T/myeloid or B/myeloid progeny (Ohmura *et al.*, 1999; Kawamoto *et al.*, 2000). Interestingly, no bi-potential T/B progenitors have been found in the FL up to now, although such a progenitor has been demonstrated at the clonal level in adult BM (Kondo *et al.*, 1997).

The inability or difficulty to detect some intermediate precursor stages in the BM, like the B/macrophage progenitor, could possibly be due to a different progress of hematopoiesis in FL and BM (Douagi *et al.*, 2002). Fetal hematopoiesis is initiated by a synchronous differentiation of a pool of multipotent cells. Thus, it leads to an enrichment of intermediate precursors derived from the first waves of stem cell differentiation at certain time points (Strasser *et al.*, 1989). In contrast, ongoing hematopoiesis in the bone marrow results in a relatively low frequency of intermediate precursors, as compared to committed and mature cell types. Thus, the failure to detect such cells might be rather of technical nature than of a systemic difference.

Distinct waves of differentiation can also be seen in T lymphopoiesis in the mouse (Itohara *et al.*, 1989; Havran and Boismenu, 1994). Interestingly, these waves result in T cells populating different anatomical sites in the adult mouse. The first T cells that appear during fetal development are γ:δ T cells using Vγ5 (also known as Vγ3 in other systems) for their γ chain rearrangement. All cells of this wave express the same rearranged γ chain without N nucleotide insertion and the same δ chain displaying, therefore, the same specificity. These γ:δ T cells populate the epidermis of the skin. Before birth generation of this subset declines and is replaced by cells expressing the next proximal Vγ gene, Vγ6. This subset reaches its maximum around birth and generation declines afterwards. Such T cells migrate to the reproductive epithelium. Later in ontogeny T cells are produced continuously and no longer in waves. After birth α:β T cells become the predominating thymocyte population, although some γ:δ T cells are still generated. However, these γ:δ T cells now exhibit more receptor diversity.

In fetal thymic organ culture (FTOC) it was shown, that Vγ5 expressing γ:δ T cells could only be generated from fetal liver HSCs, but not from HSCs isolated from adult BM. The fetal thymic environment was shown to be necessary for this differentiation process (Ikuta *et al.*, 1990).

The analysis of $Pax5^{-/-}$ mice also demonstrated differences between hematopoietic development in fetal liver and adult bone marrow (Nutt *et al.*, 1997). In the bone marrow, B cell development proceeds until the pre-B-I stage, where DJ_H at the heavy chain locus is already rearranged. This is in contrast to the fetal liver, where no B lineage committed cells (i.e. $B220^+$ cells) are detectable, suggesting a different and critical function for the Pax5 transcription factor during fetal hematopoiesis.

Furthermore, the earliest B lineage committed cells, the $B220^+CD43^+CD24/HSA^{lo}BP\text{-}1^-$ pre-pro-B cells in the bone marrow, could be further subdivided into two subsets based on cell surface expression of CD4 (Allman *et al.*, 1999). Such CD4 expressing pre-pro-B cells do not exist in the FL (Lu *et al.*, 2002). Although the $CD4^+$ pre-pro-B cells of the BM fail to develop into B cells *in vitro* (Wineman *et al.*, 1992; Kouro *et al.*, 2001), commitment to the B lineage was concluded from the detection of heavy chain germline transcripts and expression of certain B lineage associated genes (Li *et al.*, 1996; Lu *et al.*, 2002). Nevertheless, further studies on this subset demonstrated its capacity to generate dendritic cells leading to the suggestion that there is a common pathway for dendritic cell and early B cell development (Izon *et al.*, 2001).

IL-7 was shown to be a crucial factor for the development of murine B lymphocytes. The identification of its receptor complex revealed that it is composed of a private α chain, IL-7Rα (Goodwin *et al.*, 1990), and the common γ chain (γc), shared with other cytokine receptor complexes (Noguchi *et al.*, 1993). Mice lacking either IL-7 or the IL-7 receptor α chain show a severe reduction in the numbers of peripheral B cells due to a block in B cell development at the pre-pro-B cell stage ($IL\text{-}7R\alpha^{-/-}$; Peschon *et al.*, 1994) or at the pre-B-I stage ($IL\text{-}7^{-/-}$; Freeden-Jeffry *et al.*, 1995). Interestingly, for $IL\text{-}7^{-/-}$ mice it was demonstrated that B cell development takes place during fetal and perinatal life resulting in the establishment of the B1 and the MZ B cell compartment (Carvalho *et al.*, 2001). This suggests a pathway of early B lymphopoiesis that is IL-7 independent and underscores different developmental properties of FL and BM derived B cells.

A possible factor which can compensate the lack of IL-7 is thymic stromal-derived lymphopoietin (TSLP), which also uses the IL-7Rα chain for its receptor complex (Park *et al.*, 2000; Sims *et al.*, 2000; Pandey *et al.*, 2000). Only fetal-derived pro-B cells are able to respond to TSLP, although the receptor seems to be present also on the adult BM counterpart, as concluded from mRNA expression data (Vosshenrich *et al.*, 2003).

A similar situation exists with respect to T lymphopoiesis. IL-7R$\alpha^{-/-}$ mice show a profound defect in thymopoiesis in the adult thymus (Crompton *et al.*, 1998). Although total thymocyte numbers are reduced in embryos of IL-7R$\alpha^{-/-}$ mice, all the major thymocyte subpopulations, including mature single positive cells, are generated. This suggests that adult and fetal thymic progenitors have different requirements for the IL-7 receptor and presumably for the cytokine itself.

Additional support for a distinct fetal origin of B1 and MZ B cells was provided by the analysis of conditional Rag2$^{-/-}$ mice, where deletion of the *Rag2* gene at birth results in the generation of mice whose B cells are limited to these two subsets (Hao and Rajewsky, 2001).

Another difference between fetal and adult B cell development concerns the pre-BCR expression. This receptor is expressed after successful VDJ recombination of the heavy chain and seems to be important for further B cell development. Introduction of a heavy chain by transfection into a pro-B cell line results in a strong downregulation of TdT, which normally decreases sharply at the pre-B-I to pre-B-II transition (Hardy *et al.*, 2000). In the same study, two heavy chains derived from B1 cells have been identified that fail to induce downregulation of TdT. Both heavy chains, V_H81X and V_H11, usually found in B1 specificities, are known to associate poorly with the SL chain (Keyna *et al.*, 1995; Wasserman *et al.*, 1998).

Further evidence for differences in pre-BCR signaling between FL and adult BM came from experiments in which the effect of pre-BCR assembly on the proliferation of early B lineage cells was studied (Wasserman *et al.*, 1998; Hardy *et al.*, 2000). While the pre-BCR complex on isolated bone marrow cells leads to proliferation, the same pre-BCR induces an exit from cycle in fetal liver. In contrast, the V_H11 transgene that fails to assemble efficiently with the SL chain has minimal effects on the ongoing fetal liver proliferation, allowing a continued increase in cell numbers. This difference in pre-BCR receptor signaling between FL and adult could explain the differential V_H usage between the B1 and B2 repertoires (Yancopoulos *et al.*, 1984; Marshall *et al.*, 1996; Carmack *et al.*, 1990; Hardy *et al.*, 1989). V_H81X and V_H11 heavy chains are lost in the BM due to the inefficient association with the SL chain, while survival and further maturation are favored in fetal liver.

Several molecules were found to be differentially expressed during fetal and adult B cell development. One known example is the expression of MHC class II molecules, which are expressed on all mature B cells and are required for antigen presentation to CD4$^+$ T-helper cells. While MHC class II is already present on pre-B cells from the adult BM and thus

precedes the surface Ig expression, pre-B cells of the FL lack the expression of such molecules. In neonates MHC class II molecules become apparent at about the same time as IgD and CD5 are expressed on the B cells (Lam and Stall, 1994; Hayakawa *et al.*, 1994). A myosin light chain gene provides another example of late-onset expression. This internal protein is absent in precursors from the FL, in contrast to BM derived precursors (Oltz *et al.*, 1992).

Another prominent difference between fetal and adult B cell development is that fetal B cell precursors express little or no TdT during VDJ recombination of the heavy chain (Li *et al.*, 1993). Consequently, heavy chains from B cells generated in the BM usually contain N nucleotides at the V_HD and DJ_H junctions, while in junctions generated during fetal development N nucleotide addition is low or absent (Li *et al.*, 1993; Gilfillan *et al.*, 1993).

Light chains generally show no N nucleotide diversification. Although there is no strict correlation between TdT expression and TdT activity (Cherrier *et al.*, 2002), the lack of N nucleotide diversification at the heavy chain junctions is usually taken as indicator whether a B cell has originated from fetal sources or not. Early studies have shown, that receptors expressed by B1 cells only rarely have N nucleotide insertions, whereas receptors expressed by B2 cells nearly always have such insertions (Gu *et al.*, 1990; Carlsson and Holmberg, 1990; Feeney, 1990). But recent data using more sensitive methods reveal that the N nucleotide differences between B1 and B2 cells were greatly overestimated. Roughly 5% of B2 cells and 30 % of B1 cells express sequences with no N nucleotide insertion at either the V_HD or DJ_H junction, while the majority of cells in both lineages express Ig with N nucleotide insertion in at least one junction (Kantor *et al.*, 1997; Kantor *et al.*, 1995a; Kretschmer *et al.*, 2002; Kretschmer *et al.*, 2003b).

As a further consequence of the lack of TdT expression, homology-directed recombination can take place (Fig. 1-11). In lymphocytes isolated from fetal liver or from $TdT^{-/-}$ mice, V_HD and DJ_H junctions in which short stretches of nucleotides could have derived from either germline segment, were observed (Feeney, 1992).

Although the exact mechanism is not clear, it was demonstrated that short stretches of homology present on juxtaposed segments can strongly influence the outcome of rearrangement in the absence of cellular selection (Gerstein and Lieber, 1993; Chukwuocha *et al.*, 1995; Feeney *et al.*, 1994).

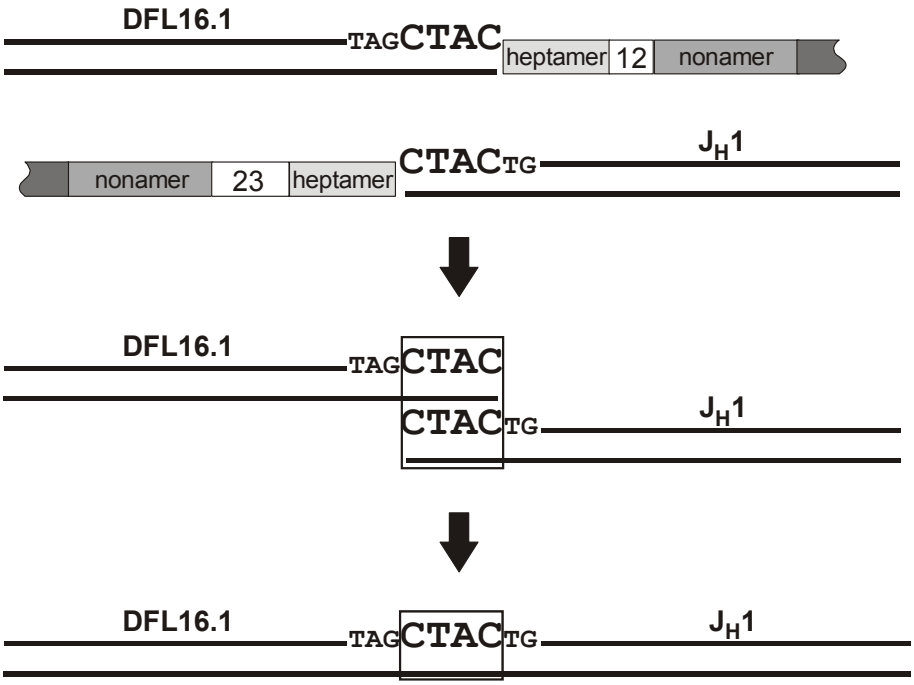


Fig. 1-11: Homology-directed recombination. As an example the homology-directed recombination between a DFL16.1 and a J_H1 gene segment is shown (modified from Gerstein and Lieber, 1993).

Interestingly, based on their germline sequences, homology-directed recombination will favor in frame, productive rearrangements of V_H7183 and Q52 genes, but out-of-frame, non-productive rearrangements of the large J558 gene family (Chukwuocha *et al.*, 1995). This provides an explanation for the finding that B1 cells often express heavy chains encoded by Q52 and V_H7183 gene families, while B2 cells mainly express Ig encoded by the J558 V_H family.

Although the absence of TdT during fetal development limits CDR3 diversity, this lack on the other hand is important for the generation of certain specificities. Forced expression of TdT during fetal life in a transgenic mouse eliminated the well-characterized T15 anti-phosphorylcholine (PC) B cell subset that is protective against pneumococcal infection. These animals became susceptible to such infections (Benedict and Kearney, 1999).

1.12. Aim of my work

As described many differences in lymphopoiesis are known between fetal and adult life up to now. In the case of B cell development the two subpopulations B1 and B2 seem to be the

result. The fetal liver was suggested as origin for B1 cells, while B2 cells originate mainly from the bone marrow. One could assume, that B cell progenitors from both organs display essential differences, and several molecules are already known to be differentially expressed. Substantial additional information should come from gene expression analysis. Therefore in this work genes differentially expressed between progenitor B cells of the fetal liver and the adult bone marrow should be investigated. This should provide further evidence for distinct progenitors and function.

The model of two distinct progenitors is not generally excepted, although the analysis of IL-$7^{-/-}$ mice (Carvalho *et al.*, 2001) and conditional $Rag2^{-/-}$ mice (Hao and Rajewsky, 2001) clearly demonstrated that the generation of B1 cells and MZ B cells takes place during fetal and perinatal life in an IL-7 independent manner. However, there was also contrasting evidence provided that the differences between B1 and B2 cells are derived solely from differences in the specificity of their receptors (Cong *et al.*, 1991; Berland and Wortis, 2002). Consequently, it was suggested that the adult bone marrow could also give rise to B1a cells.

Most of the data leading to the two different models were derived from transgenic mice or adoptive transfer experiments. To definitively answer the question, whether the adult bone marrow could also generate B1a cells, and whether B1 and B2 cells are derived from different progenitors or not, it appeared necessary to generate a mouse in which this question could be investigated under more physiological conditions. Analogous to the conditional *Rag2* knock-out mouse, a conditional *Rag1* knock-in mouse should be generated, in which B cell development could be induced at a desired time point.

Another aspect of the B cell development – differential expression of germline transcripts – should be included in this work. Germline transcription can be taken as diagnostic marker for the accessibility of a particular cluster of Ig gene segments to the recombination machinery. Characterization of germline transcripts of the murine Ig loci has so far been mainly focused on transcripts derived from the constant region cluster. Germline transcripts of V regions have not been studied to the same extent. Interestingly, transcription of germline V_H gene segments seems to be differentially regulated depending on the stage of B cell development. This has been shown in B cell lines of different developmental stages as well as in fetal liver derived B cells (Haines and Brodeur, 1998; Lennon and Perry, 1990). Differential regulation appears to depend on the distance between the DJC cluster and a particular V_H gene segment. This is supported by the fact that B cells with rearrangements at V_H regions proximal to the DJC cluster dominate fetal liver (Perlmutter *et al.*, 1985).

In former work, germline transcripts of both the κ and the λ light chain loci have been isolated. The obtained sequences should now be characterized and used for further investigation. It should be determined whether members of the V_κ cluster are activated differentially, depending on their distance from the JC cluster, or whether the whole cluster of V_κ segments is activated *in toto*. In addition, it should be investigated whether V and JC clusters of both light chain loci are regulated in a coordinate way or independently.

2. Material

2.1. Bacterial strains

2.1.1. TOP10

The *Escherichia coli* (*E. coli*) strain TOP10 (Invitrogen) was taken for the general cloning of the targeting vector. Initially chemically competent bacteria were used; later cloning steps with larger constructs were performed with electrocompetent bacteria (kind gift of Wiebke Hansen, GBF Braunschweig).

Genotype:

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

2.1.2. INV110

During the cloning of the targeting vector, ClaI sites were found methylated by the *dam* methylase and were therefore not cleavable by the corresponding enzyme. INV110 (Invitrogen) is a *dam* and *dcm* methylase-deficient *E. coli* strain and allows production of DNA that is unmethylated at the specific methylation sites. The presence of the *dam* deficiency affects INV110 in a number of ways. These bacteria have a higher mutation rate (greater than 250-fold) and a higher recombination frequency when compared to *dam*$^+$ *E. coli* strains. Therefore INV110 were only used for single transformation steps where they were absolutely required. Picking of colonies and isolation of DNA was done within one day after transformation. For the transformation chemically competent bacteria were used.

Genotype:

F' {*tra*Δ36 *pro*AB *lac*I^q *lac*ZΔM15} *rps*L (Str^R) *thr leu end*A *thi*-1 *lac*Y *gal*K *gal*T *ara ton*A *tsx dam dcm sup*E44 Δ(*lac-pro*AB) Δ(*mcr*C-*mrr*)102::Tn10 (Tet^R)

2.1.3. 294-Cre

The *E. coli* strain 294-Cre has been constructed by integrating the Cre recombinase gene into the *lacZ* locus of the strain MM294 (Buchholz *et al.*, 1996a). The expression of the Cre recombinase is under the control of the temperature-sensitive cI857 repressor. Such bacteria

can be used as test for the recombination competence of constructs that are designed for the use in Cre-mediated genomic manipulations. For this test chemically competent bacteria were used (kind gift of Anne Fleige, GBF Braunschweig).

Genotype:

$F^- \lambda^-$ *sup*E44 *end*A1 *thi*-1 *hsd*R17 *lac*Z::cI857-Cre

2.2. Cells

2.2.1. Embryonic feeder (EF) cells

Embryonic stem (ES) cells were cultured on a layer of mitotically inactive feeder cells derived from embryonic fibroblasts. These EF cells have to be G418-resistant and, therefore, were prepared from mice harboring the neomycin resistance gene. In this work IL-4 transgenic mice (Muller *et al.*, 1991) were used and EF cells were prepared from day 13 embryos. These primary feeder cells were frozen in aliquots after a first expansion and were termed EF_0.

2.2.2. Embryonic stem cells

For the generation of gene targeted mice Bruce-4 ES cells (Kontgen *et al.*, 1993) derived from C57BL/6 mice and BALB/c ES cells (Noben-Trauth *et al.*, 1996) derived from BALB/c mice were used.

2.3. The BAC (bacterial artificial chromosome) clone

For the cloning of the targeting vector a piece of genomic DNA was needed containing the Rag1 locus. Therefore the BAC clone RP23-111E15 (GenBank #AC084753), comprising the appropriate region, was ordered from the library RPCI-23 Mouse BAC II. This library has been created by Kazutoyo Osoegawa, Minako Tateno and Pieter de Jong at the Roswell Park Cancer Institute, Buffalo, New York. For the generation of the library kidney and/or brain genomic DNA from female 6 to 12 weeks old C57BL/6J mice was used, partially digested and cloned into the vector pBACe3.6 (BAC). As host the *E. coli* strain DH10B was used.

2.4. Vectors used for the construction of the targeting construct

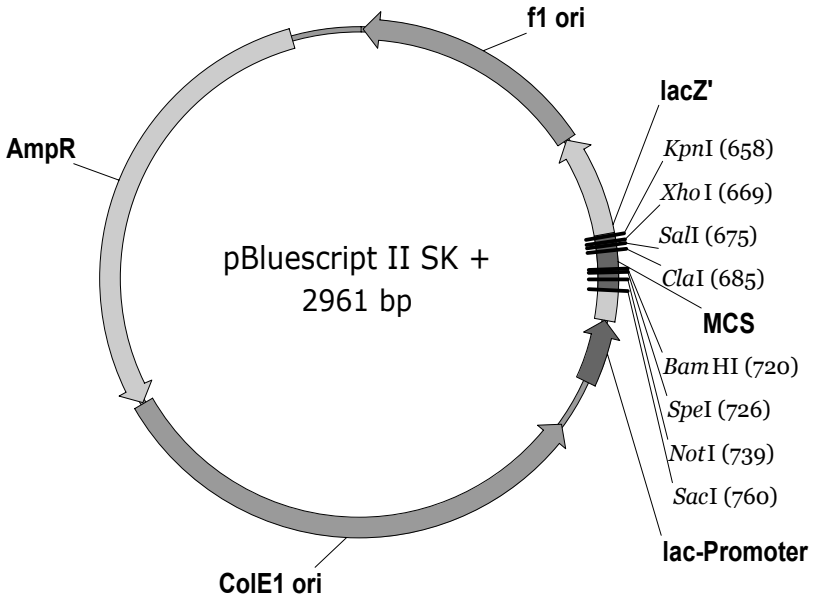


Fig. 2-1: The pBluescript® II SK + phagemid (Stratagene). pBluescript® II SK + is a phagemid derived from pUC19. The ColE1 plasmid origin of replication is used in the absence of helper phage. The Ampicillin-resistance gene (AmpR) was utilized for antibiotic selection of the vector. The multiple cloning site (MCS, 658 – 760 bp) was replaced by a modified one. The modified vector was then the basis for the cloning of the targeting vector.

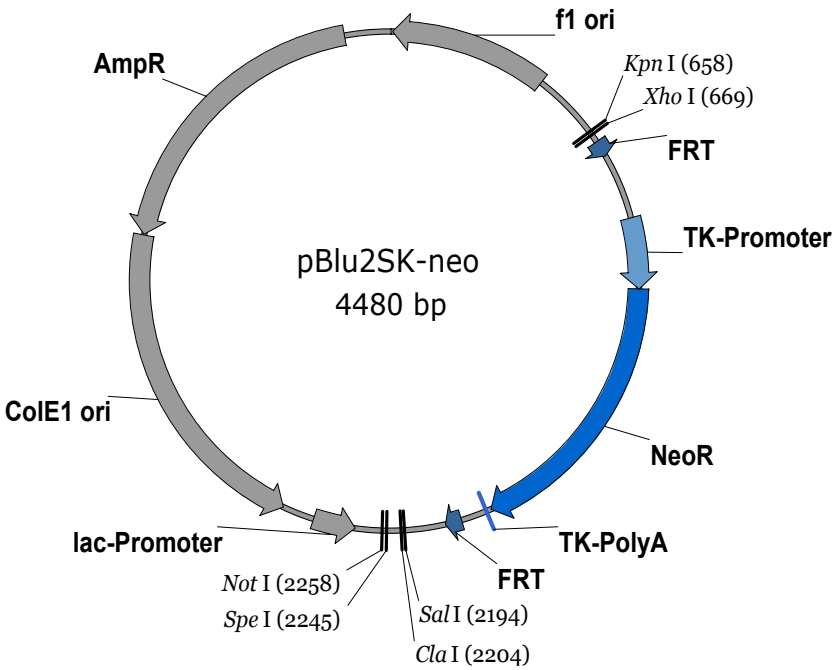


Fig. 2-2: The neomycin resistance cassette of pBlu2SK-neo. The *FRT* flanked neomycin resistance cassette was cloned XhoI/SalI into the vector pBluescript® II SK + afterwards termed pBlu2SK-neo (kind gift of Werner Müller, GBF Braunschweig). The neomycin resistance gene (NeoR) is controlled by the herpes simplex virus (HSV) thymidine kinase (TK) promoter and contained also the TK polyA signal.

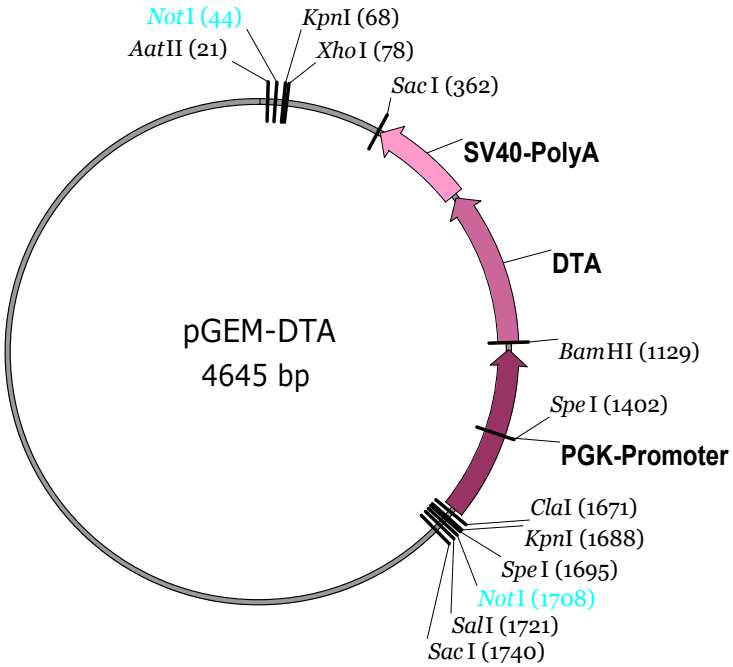


Fig. 2-3: The DTA (diphtheria toxin A) cassette of pGEM-DTA. The DTA cassette was PCR amplified out of the vector pROSA26-1 (see http://www.fhcrc.org/labs/soriano) and cloned into the vector pGEM-T Easy (Promega) afterwards termed pGEM-DTA (kind gift of Thorsten Buch, University of Cologne). The diphtheria toxin subunit is controlled by the PGK-promoter and contains the SV40-PolyA signal. Restrictions sites used for the excision of the cassette are marked in blue.

2.5. Mice

The injection of recombinant ES cells into blastocysts was carried out in Braunschweig (Experimental immunology, GBF Braunschweig) or in Cologne (Center for Mouse Genetics, University of Cologne). Also subsequent breeding was carried out in Braunschweig or Cologne. BALB/c and C57BL/6 wild-type mice were obtained from Harlan Winkelmann GmbH (Braunschweig), Charles River (Cologne) or from internal breeding. CD1 mice (Braunschweig), F1 mice (F1 generation of BALB/c x C57BL/6) and CB20 mice (both Cologne) were derived from internal breeding.

Donor embryos for blastocyst injection were obtained from untreated CB20 or hormone-stimulated C57BL/6 mice at day 3.5 and used in a way that allowed chimerism to be detected by coat-color (i.e. CB20 for C57BL/6 ES cells and C57BL/6 for BALB/c ES cells). Pseudo-pregnant F1 or CD1 mice were used as foster mothers.

For the isolation of adult bone marrow cells, female BALB/c mice of an age of 7 weeks were taken. BALB/c fetuses of day 17 of gestation were obtained by timed pregnancies, the day of appearance of a vaginal plug being taken as day 0.

2.6. Antibodies

The following antibodies were used for the isolation of B cell precursors by cell sorting: the monoclonal antibody against CD19 (1D3, PharMingen) coupled with APC and an antibody against c-kit (ACK-4). The antibody against c-kit was protein G purified from concentrated hybridoma supernatants and coupled to biotin following standard protocols. The biotinylated antibody was revealed by streptavidin-R-PE (Southern Biotechnology Associates).

2.7. Oligonucleotides

Synthetic oligonucleotides required for cloning were purchased from MWG (HPSF purification). Oligonucleotides for polymerase chain reaction (PCR) and sequencing were obtained from Invitrogen (standard purification) and were controlled with the program OLIGO® (Version 4.04-s, National Biosciences) for duplex formation and hairpin structures.

2.7.1. Oligonucleotides used for cloning

Annealed oligonucleotides were used for the introduction of short stretches of DNA into the targeting vector. The oligonucleotides are shown here in the annealed double-stranded form. Black and pink nucleotides represent the sequence of the oligonucleotides, while blue nucleotides only serve to elucidate the restriction sites. Pink nucleotides do not reconstitute a given recognition motif which leads to the destruction of the restriction site after integration of the annealed oligonucleotides into compatible sites.

NeoR cassette mod1 (KpnI-NheI-10 bp-NheI-XhoI)

NheI-site-for 5'-GGTACC^GCTAGC^ACGCTTACCA^GCTAGC^CTCGAG-3'
NheI-site-rev 3'-CCATGG^CGATCG^TGCGAATGGT^CGATCG^GAGCTC-5'

NeoR cassette mod2 (SalI-2 bp-SpeI-XhoI-NheI-NotI)

SpeI-site-for 5'-GTCGAC^AT^ACTAGT^CTCGAG^GCTAGC^GCGGCCGC-3'
SpeI-site-rev 3'-CAGCTG^TA^TGATCA^GAGCTC^CGATCG^CGCCGGCG-5'

new MCS (SacI-NotI-BamHI-AatII-~~KpnI~~)

MCS-for 5'-GAGCTC^GCGGCCGC^GGATCC^GACGTC^TGTACC-3'
MCS-rev 3'-CTCGAG^CGCCGGCG^CCTAGG^CTGCAG^ACATGG-5'

loxinv (~~KpnI~~-LoxP-NdeI-ClaI-2 bp-KpnI-SpeI-PxoL-~~ClaI~~)

LoxP-i-for 5'-GGTACT^ATAACTTCGTATAGCATACATTATACGAAGTTAT^CATATG
LoxP-i-rev 3'-CCATGA^TATTGAAGCATATCGTATGTAATATGCTTCAATA^GTATAC

^ATCGAT^AT^GGTACC^ACTAGT^ATAACTTCGTATAATGTATGCTATACGAAGTTAT
^TAGCTA^TA^CCATGG^TGATCA^TATTGAAGCATATTACATACGATATGCTTCAATA

^ACCGAT-3'
^TGGCTA-5'

2.7.2. Oligonucleotides used for PCR

HYB-1-FOR: 5'-AGG GTC GAT AAA AGT AAG CAA CAC AGA-3'
HYB-1-REV: 5'-TCC CAT GAG ATC ACA GAA AGA AAA AGA-3'

HYB-3-FOR: 5'-CCC TTG GTT TGC ACT GAC TTT-3'
HYB-3-REV: 5'-TTC CGG GAG ATT GGT TGT T-3'

HYB-5-FB: 5'-GGT GAA CCG GGA GAA AGT GA-3'
HYB-5-REV: 5'-TCA TAA TTT TAA TCA CAG GTT TTG-3'

NEO-FOR: 5'-ACT GGG CAC AAC AGA CAA TCG GCT-3'
NEO-REV: 5'-TAT TCG GCA AGC AGG CAT GCG CAT-3'

RAG1-FOR-1: 5'-GAC AAA GCA GTT CAC CAA GCC AG-3'
RAG1-REV-1: 5'-GCG TGG TTG AGC ACA GTT TTT AGT T-3'

RAG1-FOR-2: 5'-CCC ACA GTC AGG TCT ACT TCC CAA-3'
RAG1-REV-2: 5'-GAC AGC CTT CAC ATC TCC ACC TTC T-3'

RAG1-S3: 5'-AGC TAT CAC TGG GAG GCA GAT TTT-3'
RAG1-REV-3: 5'-CGT ATC CAG TGC CCC TGA AGA-3'

2.7.3. Oligonucleotides used for semi-quantitative RT-PCR

β-actin:	bActfor;	
	bActrev:	both Engel *et al.*, 1999
GAPDH:	GAPDH for:	5'-ATC TTC TTG TGC AGT GCC AGC-3'
	GAPDH rev:	5'-ACT CCA CGA CAT ACT CAG CAC C-3'
Vλ1/2:	VL1+2FORLEA:	5'-TGA ATT ATG GCC TGG A(CT)T TCA C-3'
	Vλ1rev:	5'-GCC ACC TGT TAA GAA GAT GGT AGT TA-3'
Vλx:	Vx-for:	5'-CAT TAT GGC CTG GAC TCC TC-3'
	Vx-rev:	5'-TCT TCA GGC TGG ATG TTG G-3'
$\lambda 1^0$:	Stλ1for2;	
	Cλ1R3:	both Engel *et al.*, 1999
Vκ02:	Vκ2-L-for:	5'-ATG ATG AGT CCT G(CT)C CAG TTC CTG-3'
	Vκ02-FR3REV-1:	5'-CTC CCA AAT C(CT)T CAG CCT CCA CTC T-3'
Vκ21:	Vκ21L-1:	5'-CTG CTA TGG GTG CTG CTG CTC TG-3'
	Vκ21-FR3REV-1:	5'-GGA TGG ATG TTG AGG (CG)TG AAG TCT G-3'
$\kappa^0$0.8:	0.8 K0FOR;	
	K0REV:	both Grawunder *et al.*, 1995b
$\kappa^0$1.1:	1.1 K0FOR:	5'-CAT GTG AAG TGA AAT GGC TGT AGC CTA ATG-3' (Grawunder *et al.*, 1995b)
	K0REV:	see above

2.7.4. Oligonucleotides used for sequencing

NEO-R0:	5'-TGT GTT TCC CTC TCA ATC AAG TCT TAG-3'
NEO-R1:	5'-ACG AGG AAG CGG TCA GCC CAT T-3'
NEO-R2:	5'-ATC ATC CTG ATC GAC AAG ACC G-3'
NEO-R3:	5'-CGG ACA GGT CGG TCT TGA CAA A-3'
NEO-R4:	5'-ACC TGC GTG CAA TCC ATC TTG TTC-3'
RAG1-FOR-0:	5'-AGG GCA TCA ATC AGT TAT TTC CAA A-3'
RAG1-FOR-1:	see above
RAG1-FOR-2:	see above
RAG1-S1:	5'-GAT ATG CGA ACA CAT TCT GGC TGA T-3'
RAG1-S2:	5'-GCC TCG CCA GCA TCT CCT GTC-3'

RAG1-S3: see above
RAG1-S3.5: 5'-TGG AAT TAT TGA TGG GCT GTC TGG-3'
RAG1-S4: 5'-AGT GGT GGT AAA GGA GTC TTG CGA-3'
RAG1-S5: 5'-TCT TCA GGG GCA CTG GAT ACG-3'
RAG1-S6: 5'-CAT AGA TGC GCT TCA CTG TGA CA-3'
RAG1-S7: 5'-GAA ACC CGT GTG GCG CTC TT-3'
RAG1-S8: 5'-ACC TCC AGA AGT TTA TGA ATG CTC AT-3'
RAG1-SLOX: 5'-GAA GAA ATA AAC AAC CAA TCT CCC G-3'
RAG1-SLOX-2: 5'-CTC TGT GCA TCC CTG GTC CCC T-3'
RAG1-REV-3: see above

M13 Forward (-20): 5'-GTA AAA CGA CGG CCA G-3' (Invitrogen)
M13 Reverse: 5'-CAG GAA ACA GCT ATG AC-3' (Invitrogen)

2.8. Culture media

2.8.1. Culture media for bacteria

LB medium

1% (w/v) Bacto™ Tryptone (BD)

1% (w/v) Bacto™ Yeast Extract (BD)

0.5% (w/v) NaCl

diluted in H_2O and autoclaved.

LB plates with ampicillin

For the generation of agar plates 1.5% (w/v) of Bacto™ Agar (BD) was added to the LB medium before autoclaving. After autoclaving the medium and cooling down to 50°C, the ampicillin stock solution was diluted in the medium 1:1000 resulting in an end concentration of 50 µg/ml. 25 ml of this medium were used per plate. The plates were stored at 4°C after solidification.

Ampicillin stock solution

5% (w/v) Ampicillin (sodium salt, SIGMA) dissolved in 70% ethanol and stored at –20°C.

2.8.2. Culture media for cells

EF cell medium

500 ml Dulbecco's MEM with Glutamax-1 (Gibco/Invitrogen)

60 ml Fetal bovine serum (Biochrom KG)

6 ml Sodium pyruvate MEM 100MM (Gibco/Invitrogen)

6 ml Penicillin-Streptomycin (Gibco/Invitrogen)

were mixed and stored at 4°C.

ES cell medium

500 ml Dulbecco's mod eagle medium (Gibco/Invitrogen)

75 ml Foetal bovine serum (performance tested, Gibco/Invitrogen)

6 ml Sodium pyruvate MEM 100MM (Gibco/Invitrogen)

6 ml Penicillin-Streptomycin (Gibco/Invitrogen)

6 ml L-Glutamine 200MM (Gibco/Invitrogen)

6 ml MEM non-essential amino acids (Gibco/Invitrogen)

1.2 ml LIF (supernatant of the cell line CHO 8/24 720 LIF D(.1))

0.6 ml 0.1 M 2-Mercaptoethanol

were mixed and stored at 4°C.

0.1 M 2-Mercaptoethanol

50 µl 2-Mercaptoethanol (SIGMA) dissolved in 7 ml PBS (see 3.2.2.), filter sterilized and stored as 0.6 ml aliquots at –20°C.

2.9. RNA from R2-bfl cells

The *Rag2* deficient, bcl-2-transgenic pro-B cell line R2-bfl was grown on the stromal cell line ST-2 in the presence of recombinant IL-7 as described previously (Grawunder *et al.*, 1995b). Differentiation was induced by removal of IL-7 and different cultures of cells were cultivated

for additional one to five days. Total RNA was isolated from the cells using Trizol reagent (Gibco) according to the manufacturer's protocol (kind gift of Dr. Holger Engel).

2.10. Cloned germline transcripts

Amplification products of germline transcripts from both light chain loci were obtained by 3'-RACE (rapid amplification of cDNA ends; Dr. Holger Engel, unpublished and Sandra Düber, diploma thesis, TU Braunschweig 2001) using total RNA from R2-bfl cells differentiated for four days in the absence of IL-7 and the SMART RACE cDNA Amplification Kit (Clontech). The following V gene family specific primers were used for the 3'-RACE:

Vκ01L-1: 5'-CCT GTT AGG CTG TTG GTG CTG ATG-3'
Vκ2-L-for: see above
Vκ04/05L-2: 5'-GGA TTT TCA GGT GCA GAT TTT CAG C-3'
Vκ08L-1: 5'-GCA AGA TGG ATT CAC AGG CCC AG-3'
Vκ08L-2: 5'-CAA GAT GGA ATC ACA GAC TCA GG-3'
Vκ09/10L-1: 5'-CAG GAC TCA GCA TGG ACA TGA GGG-3'
Vκ09/10L-2: 5'-GCT CAG TTC CTT GGT CTC CTG TTG-3'
Vκ12/13L-1: 5'-GTT GCT GCT GCT GTG GCT TAC A-3'
Vκ19/28L-1: 5'-GGG CAT CAA GAT GGA (AG)TC ACA GA(CT) TC-3'
Vκ21L-1: see above
Vκ22L-1: 5'-GGA GCA GGA TGG AGT TTC AGA CC-3'
Vκ24/25L-2: 5'-GAT GCT TGT GCT CTG GAT TCC TG-3'
Vκ38cL-1: 5'-CGT CTA TTC AGT TCC TGG GGC TC-3'
VκDVL-1: 5'-GCC TCA GAA AGA AAA TCG TGA ACC-3'
Vλ1forLea: 5'-TGA ATT ATG GCC TGG ATT TCA-3'
Vλ2forRACE: 5'-CTT GGT TTG TGA ATT ATG GCC TGG AC-3'
VxforLeader: 5'-TGC ATT ATG GCC TGG ACT CCT CTC-3'.

In some cases a nested-PCR was performed using the following V_κ gene family specific primers derived from the first framework region:

Vκ01FR-1: 5'-TGA TGA CCC AAA CTC CAC TCT CC-3'
Vκ08FR-1: 5'-GGA GA(AG) AAG GT(CT) ACT ATG AGC TG-3'
Vκ21FR-1: 5'-ACA CAG TCT CCT GCT TCC TTA GC-3'

As reverse primer a nested universal primer supplied with the SMART RACE kit was used.

PCR products of the expected size were cloned using the TOPO TA cloning kit (Invitrogen). Several clones were picked and sequenced. Sequences were analyzed using Sequencher software (Gene Codes Corporation) and database comparison with published sequences using advanced BLAST search and IgBlast (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/).

3. Methods

3.1. Molecular biological methods

3.1.1. Isolation of plasmid DNA

The isolation of plasmid DNA for analytical and preparative purposes was performed with the "GFX Micro Plasmid Prep Kit" (Amersham Biosciences). The isolation of plasmid DNA for transfections was carried out with the "QIAGEN® Plasmid Maxi Kit" (QIAGEN). Both purifications were done according to the manufacturer's protocols.

3.1.2. Isolation of BAC DNA

The BAC DNA was isolated using the "NucleoBond®PC500 kit" (Macherey-Nagel) according to the manufacturer's protocol. To avoid shearing of the BAC DNA, the bacterial lysate was filtrated and not centrifuged for clarification.

3.1.3. Cleavage of DNA with restriction enzymes

Restriction endonucleases recognize short specific DNA sequences, mostly 4 to 8 nucleotides long, and cleave the DNA in this region. Cleavage of DNA with restriction enzymes was used for the cloning of the targeting vector and for the linearization of the targeting construct before transfection. Generally, this cleavage was performed in 20 µl volume, but sometimes the volume was scaled up. The reaction was carried out using optimal buffer and temperature conditions for each enzyme according to the manufacturer's protocol. For the digestion of genomic DNA for Southern blot 1 mM spermidine, 1 mM DTT and 50 µg/ml RNaseA were added to the mix.

3.1.4. Dephosphorylation of DNA

If vector DNA is cleaved with only one restriction enzyme, two compatible ends are generated, that can re-ligate. To minimize this option it is possible to dephosphorylate the vector. For this hydrolysis of 5'-phosphate residues calf intestine alkaline phosphatase (CIAP, MBI Fermentas) was used according to the manufacturer's protocol.

3.1.5. Phenol/chloroform extraction

Clean DNA of high quality was needed for the transfection of ES cells. To remove enzyme and salts after the restriction digestion for linearization, extraction with phenol/chloroform was performed.

An equal volume of phenol/chloroform (1:1) was added to the DNA. After vortexing for 1 minute, the phases were separated by centrifugation for 10 minutes (10.000 x g, RT). The upper phase was transferred to a new tube and an equal volume of chloroform was added. After vortexing for 1 minute, the phases were separated again by centrifugation for 10 minutes (10.000 x g, RT). This chloroform extraction step was repeated. Then the DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. After 30 minutes of centrifugation (10.000 x g, RT), the supernatant was removed. The tube was filled completely with 70% of ethanol and stored over night at 4°C. This last step should sterilize the DNA.

3.1.6. Annealing of oligonucleotides

The two oligonucleotides that should anneal were diluted with water to an end concentration of 100 pmol/µl. 10 µl of each oligonucleotide were mixed in a tube and heated for 5 minutes to 95°C. Then the tube was placed in a cup of water at 60°C and tube and water were allowed to cool down to room temperature. 1 µl of the mix was taken later for the ligation.

3.1.7. Gel electrophoresis in agarose gels

DNA is negatively loaded in aqueous solutions due to the ionization of the phosphate residues. If an electric field is present, it is possible to separate DNA fragments corresponding to their size in a gel matrix.

In this study gels were used at 0.8 and 2% concentration of agarose (SeaKem® LE Agarose, Cambrex). The size of the DNA fragments was estimated by comparison with DNA markers. The gels were running in 1x TAE puffer mostly at 160 V. DNA fragments were stained with ethidium bromide and visualized using UV light. Documentation was performed using the Herolab GmbH software.

50x TAE: 242 g Tris

57.1 ml Acetic acid

100 ml 0.5 M EDTA (pH 8.0)

filled up with H_20 to 1 liter and autoclaved.

DNA marker: SmartLadder SF, 100bp-1kb (Eurogentec)

GeneRuler™ 1kb DNA Ladder, 250bp-10kb (MBI Fermentas)

Mixture of *Hin*dIII digested λ DNA and *Bsu*RI digested ΦX174 DNA,

72bp-23kb (MBI Fermentas)

3.1.8. Gel extraction

The extraction of DNA fragments from agarose gels for the later ligation was performed with the "QIAquick Gel Extraction Kit" from QIAGEN according to the manufacturer's protocol. With this kit fragments from 70 bp to 10 kb can be processed. If the DNA fragment was required in a higher concentration the "MinElute™ Gel Extraction Kit" from QIAGEN was used. Here it is possible to elute the DNA in a smaller volume of buffer (10 µl). With this kit only fragments in the range from 70 bp to 4 kb can be processed.

3.1.9. Ligation of DNA

DNA fragments and linearized vectors with cohesive or blunt termini can be joined by the use of ligases. The used T4 DNA ligase (MBI Fermentas) catalyses the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in double-stranded DNA. Vector DNA and the DNA fragment were optimally used in a molecular ratio of 1:10, to minimize the chance of vector re-ligation. The ligation was performed according to the manufacturer's protocol. For cloning steps with cohesive ends and large amounts of DNA fragment, the reaction was incubated 1 hour at room temperature. More difficult cloning steps (blunt end ligation or a low amount of DNA fragment) were incubated at 16°C over night.

3.1.10. Transformation of bacteria

Different methods of transformation were carried out to introduce plasmid DNA into bacteria.

3.1.10.1. Transformation of chemically competent *E. coli* (Invitrogen)

The competent bacteria were thawed on ice, mixed with 10 µl of the ligation reaction or with 1 µl of plasmid DNA by gently tapping and were incubated for 30 minutes on ice. After a subsequent incubation for 30 to 45 seconds at 42°C, the bacteria were placed back on ice. 250 µl of SOC medium (supplied with the bacteria) were added to the bacteria and the suspension was shaken for one hour at 37°C. Afterwards the bacteria were plated on LB plates containing ampicillin (50 µg/ml) and the plates were incubated at 37°C over night.

On the next day, single colonies were picked and cultivated in LB medium containing ampicillin (100 µg/ml). The liquid cultures were shaken at 180 rpm and 37°C for 6 to 8 hours. Afterwards the clones were analyzed by plasmid isolation and restriction digestion.

In case of the 294-Cre bacteria the cultivation of the liquid cultures was carried out over night at 27°C.

3.1.10.2. Electroporation of *E. coli*

The electrocompetent bacteria were thawed on ice, mixed with 2 µl of ligation reaction by gently tapping and incubated for 1 minute on ice. The suspension was transferred into a cuvette and electroporation was carried out (parameters 2.5 kV, 25 µF, 200Ω) with a Gene Pulser (Biorad). Immediately 500 µl of SOC medium was added to the cuvette. The suspension was transferred to a tube and was shaken for 1 hour at 37°C before plating on LB plates containing ampicillin (50 µg/ml). The plates were incubated at 37°C over night. Picking of colonies was performed as described above.

3.1.11. Long-term storage of bacteria

For the long-term storage of bacteria 700 µl of a fresh bacterial culture were mixed with 300 µl of 50% glycerine and stored at –80°C.

50% glycerin: 50% (v/v) glycerin in H_20, filter sterilized and stored at 4°C.

3.1.12. Determination of nucleic acid concentrations

The nucleic acid concentration was determined after a suitable dilution of the DNA or RNA probe in TE (see 3.1.17.1.) through measurement of the extinction at 260 nm. (OD_{260}=1 correspond for DNA to a concentration of 50 µg/ml, for RNA to a concentration of 40 µg/ml, respectively.) The photometer (BioPhotometer, Eppendorf) was normalized with TE before.

3.1.13. DNA sequencing

The sequencing of DNA was carried out with the "ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit" (PE Biosystems), based on the chain-termination method of Sanger (Sanger *et al.*, 1977). This kit contains a mixture of ddNTPs coupled with different fluorescent dyes for the termination, dNTPs for the extension, AmpliTaq® DNA Polymerase FS and reaction buffer. The mixture was ready to use (premix). The reaction was performed in 200 µl reaction tubes (0.2 ml Micro-Strips & Caps, Abgene® House).

Reaction mix:	2 µl	Premix
	1.6 pmol	Primer
	60-360 ng	PCR product
or	0.6-1.2 µg	Plasmid DNA
	H_20 ad 11 µl	

The following program was used on the thermocyler (PCR Express, Hybaid): 94°C for 60 s, 25 cycles at 94°C for 60 s, 52°C for 20s, and 60°C for 4 min.

After the reaction, the DNA was precipitated to remove primers, salts and non-incorporated nucleotides. Therefore 10 µl water, 20 µl 2 mM $MgCl_2$ and 60 ml absolute ethanol were added to the reaction and mixed by inverting the tubes ten times. After 15 minutes incubation at room temperature, the precipitate was spun down (45 min, 1420 x g, RT) and the supernatant was removed by a subsequent centrifugation with inverted open tubes (1 min, 188 x g). Afterwards the DNA was washed with 100 µl of 70% ethanol. The ethanol was removed after a further centrifugation step (45 min, 1420 x g, RT) as described above. Residual ethanol

was evaporated on a thermocycler by incubation at 65°C for 1 to 2 minutes. The probes were stored at –20°C until they were loaded on the sequence gel and analyzed with an automatic DNA sequencer (ABI PRISM 377, PE Applied Biosystems).

The obtained sequences were evaluated and compared with known sequences using the program Sequencher™ (Version 3.1.1, Gene Codes Corporation). Sometimes a database comparison was performed using Advanced BLAST search and IgBLAST (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/).

3.1.14. Polymerase chain reaction (PCR)

The polymerase chain reaction allows the selective amplification of DNA sequences of low abundance, present in a heterogeneous mixture. During the reaction the region flanked by the primers is exponentially amplified.

Reaction mix:	0.5 µl	Primer 1 (10 pmol/µl)
	0.5 µl	Primer 2 (10 pmol/µl)
	2 µl	10x PCR buffer (Perkin Elmer)
	2 µl	Nucleotide mix (2 mM each nucleotide)
	13.9 µl	H_2O
	0.1 µl	AmpliTaq Gold™ (5U/µl)
	1 µl	Template DNA

The reaction was carried out in 200 µl reaction tubes (0.2 ml Micro-Strips & Caps, Abgene® House). Following programs were used on the thermocyler (PCR Express, Hybaid):

Program	Denaturation	Annealing	Extension	Cycles
HYB/H	94°C 20s	55°C 30s	72°C 30s	30
HYB/L	94°C 20s	50°C 30s	72°C 30s	30
NEO	94°C 20s	58°C 30s	72°C 30s	30
RAG	94°C 30s	60°C 60s	72°C 60s	30

Each program was started by a 10 minutes activation step at 95°C for the hotstart polymerase and finished by 7 minutes final extension at 72°C.

3.1.15. DNase treatment

RNA preparations could contain also contaminating genomic DNA. This potentially contaminating DNA was therefore removed on "RNeasy Mini Kit" spin column (QIAGEN) using the RNase-free DNase set (QIAGEN) according to the manufacturer's protocol.

3.1.16. Semi-quantitative reverse transcriptase PCR (RT-PCR)

A special form of the PCR is the RT-PCR. cDNA is used here as template. This cDNA had to be generated from mRNA by reverse transcription. Semi-quantitative RT-PCR allows measuring the relative differences in transcript levels between different samples. The simplest way of performing such analysis is to determine the amounts of PCR product during the exponential phase of the PCR but before the plateau phase. Therefore serial dilutions of the cDNA are tested in the PCR. The amount of cDNA and efficiency of reaction were controlled using primers specific for housekeeping genes.

For the preparation of cDNA, 1 µg of total RNA was reverse transcribed using Superscript II (Gibco/Invitrogen) and oligo $d(T)_{12-18}$ primer (Amersham Biosciences) according to the protocol supplied with the reverse transcriptase. As RNase inhibitor RNasin ribonuclease inhibitor (Promega) was used. The cDNA was diluted to 100 µl, which was termed the undiluted sample, and further serially diluted four-fold. 1 µl of these dilutions were taken as template for PCR. The PCR reaction was performed in a 20 µl volume as described above.

Following programs were used on the thermocyler (PCR Express, Hybaid):

Program	Denaturation	Annealing	Extension	Cycles
β-actin	94°C 20s	55°C 30s	72°C 30s	24
GAPDH	94°C 20s	58°C 30s	72°C 30s	26
Lambda	94°C 20s	52°C 30s	72°C 30s	32
V-Kappa	94°C 20s	62°C 30s	72°C 30s	32
C-Kappa	94°C 20s	65°C 30s	72°C 30s	32

Each program was started by a 10 minutes activation step at 95°C for the hotstart polymerase and finished by 7 minutes final extension at 72°C.

3.1.17. Preparation of genomic DNA

3.1.17.1. Preparation of genomic DNA from tail biopsies

The piece of the mouse tail (about 1 cm) was placed in a tube with 720 µl of tail buffer and 30 µl of proteinase K. The tube was placed into a thermomixer and shook over night at 54°C. The next morning hairs and bones were spun down (10 min, full speed, RT) and the supernatant was transferred into a new tube containing 600 µl 2-propanol. It was mixed gently but thoroughly by inverting several times until a flocculent precipitate appeared. After five minutes of centrifugation (10.000 x g), the supernatant was removed and the pellet was washed with 500 µl 70% ethanol. After an additional centrifugation the supernatant was removed again and the pellet was dried at room temperature. After drying, 100 µl of TE 10/1 was added and the DNA was dissolved by shaking for 3 to 12 hours at 54°C with a thermomixer. Afterwards the tubes were vortexed for 5 s and the DNA was stored at 4°C.

Tail buffer (Laird):	100 mM Tris HCl (pH 8.5)
	5 mM EDTA (pH 8.0)
	200 mM NaCl
	0.2% SDS
Proteinase K:	Proteinase K (SIGMA) in H_20 (10 mg/ml) stored at –20°C.
TE 10/1:	10 mM Tris (pH 7.6)
	1 mM EDTA

3.1.17.2. Preparation of genomic DNA from ES cells

It is possible to prepare the DNA of ES cells directly in the 96 well plates and do the cleavage with restriction enzymes afterwards in the same plates. The plates with the confluent ES cells were washed twice with PBS or the already washed and frozen plates were allowed to warm up to room temperature. To each well 50 µl of lysis buffer was added. The plates were wrapped with parafilm and transferred into a pre-warmed, humidified chamber of 56°C. The

lysis was performed over night. The next day the humidified chamber was allowed to cool at room temperature for 1 hour. Then 100 µl of absolute ethanol was added to each well of the plate and DNA precipitate became visible. After one hour the ethanol was removed by inverting the plate and carefully draining the wells on paper towels. The DNA should remain attached to the plastic. Then the plates were washed three times with 100 µl of 70% ethanol, carefully draining the wells after each washing step. Afterwards the wells were dried at room temperature and were ready for the restriction digestion.

Lysis puffer: 10 mM Tris HCl (pH 7.5)

10 mM NaCl

10 mM EDTA

0.5% Sarcosyl

0.4 mg/ml Proteinase K (added fresh each time)

3.1.18. Southern blot

In a Southern blot DNA restriction fragments are separated in an agarose gel and transferred (blotted) to a membrane. Hybridization of a radioactive labeled DNA probe to the immobilized target sequence allows the detection of these hybrids due to the radioactivity. Southern blot analysis is a very sensitive method routinely used in the analysis of gene structure.

3.1.18.1. Alkaline transfer

One method for the capillary transfer of DNA to membranes is the alkaline transfer. In contrast to the original protocol of Southern (Southern, 1975), only one solution is used for denaturation and transfer of the DNA.

The DNA restriction fragments were separated in an agarose gel at low voltage in the presence of ethidium bromide. Then a picture under UV light was taken including a fluorescent ruler. The size ladder was marked by punching little holes into the gel with a yellow tip. Then the gel was agitated in 0.25 N HCl for approximately 10 minutes for depurination. Afterwards the DNA was denatured by soaking the gel in 0.4 N NaOH for

30 minutes. The positively charged nylon membrane (Gene Screen Plus Hybridization Transfer Membrane, NEN™Life Science Products) was cut to exactly the same size as the gel. It was pre-wetted in distilled water for a few seconds and equilibrated for 15 minutes in 0.4 N NaOH. Afterwards a downwards capillary blot according to Fig. 3-1 was set up using 0.4 N NaOH as transfer solution.

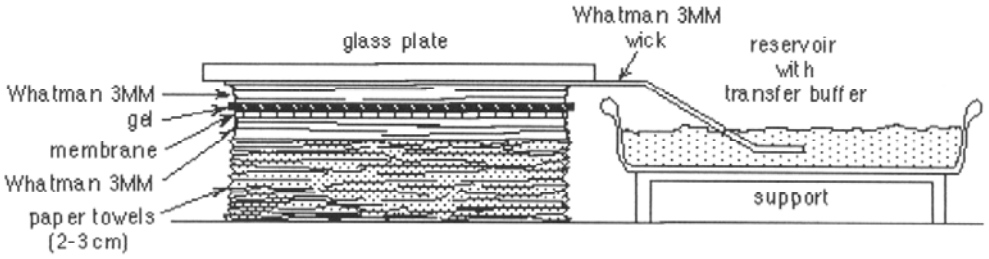


Fig. 3-1: Set-up of a downwards capillary blot.

After blotting over night, the slots of the gel and the size ladder were marked on the membrane. Afterwards the membrane was washed with excess 2x SSC for 1-2 minutes. The DNA was fixed on the membrane by drying the membrane 1-2 hours at 80°C.

20x SSC stock: 3 M NaCl

0.3 M Tri-sodiumcitrate

pH 7.2, filtered.

3.1.18.2. Labeling of the probe

The DNA probe was labeled using the "Ladderman™Labelling Kit" (TaKaRa) according to the manufacturer's protocol. With this kit the probe was labeled with [α-^{32}P] dCTP using random nonamers. After labeling, non-incorporated nucleotides were removed using ProbeQuant™ G-50 Micro Columns (Amersham Biosciences). After that 35 µl of sonicated salmon sperm DNA (10 mg/ml) were added to the probe and the mix was heated at 95°C for 5 minutes. Thereafter the probe was ready for hybridization.

3.1.18.3. Hybridization and detection

Hybridization was performed using QuikHyb® Hybridization Solution (Stratagene). This solution reduces the time required for hybridization from customary 12-24 hours down to 1-2 hours.

Prior to prehybridization the membrane was briefly dipped in deionized water to remove excess salt from the membrane. Then the membrane was prehybridized for at least 30 minutes at 65°C in a glass bottle in a rotating hybridization oven using the QuikHyb® Hybridization Solution. Afterwards the labeled probe was added to the solution and it was hybridized for at least one hour.

After hybridization the membrane was washed twice shortly with 2x SSC/0.1% (w/v) SDS at room temperature. Then the membrane was washed twice with 0.1x SSC/0.1% (w/v) SDS at 58°C for 15 minutes. Afterwards the membrane was wrapped in a plastic wrap and placed on a Kodak BIOMAX MS film with an intensifying screen at –80°C. Exposure was performed over night. The film was developed the next morning using an automatic developer machine (Curix 60, Agfa).

3.1.19. Generation of cRNA and microarray hybridization

High-density oligonucleotide arrays allow the parallel analysis of expressed mRNA of a large number of genes (Lockhart *et al.*, 1996; Lipshutz *et al.*, 1999). With the Affymetrix GeneChip MG-U74Av2 used for hybridization ~12.000 genes and expressed sequence tags (ESTs) can be tested.

Total RNA from 10^5 sorted $CD19^+c\text{-}kit^+$ pre-B cells was isolated using the "RNeasy Kit" (QIAGEN). Quality and integrity of the total RNA was controlled by running all samples on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). For RNA amplification (Fig. 3-2) the first round was done according to the protocol provided by Affymetrix without biotinylated nucleotides using the Promega P1300 RiboMax Kit (Promega) for T7 amplification. For the second round of amplification the precipitated and purified RNA was converted to cDNA primed with random hexamers (Pharmacia). Second strand synthesis and probe amplification were done as in the first round with two exceptions: An incubation with RNase H preceded the first strand synthesis to digest the aRNA, and the T7T23V oligonucleotide for initiation of the second strand synthesis was used.

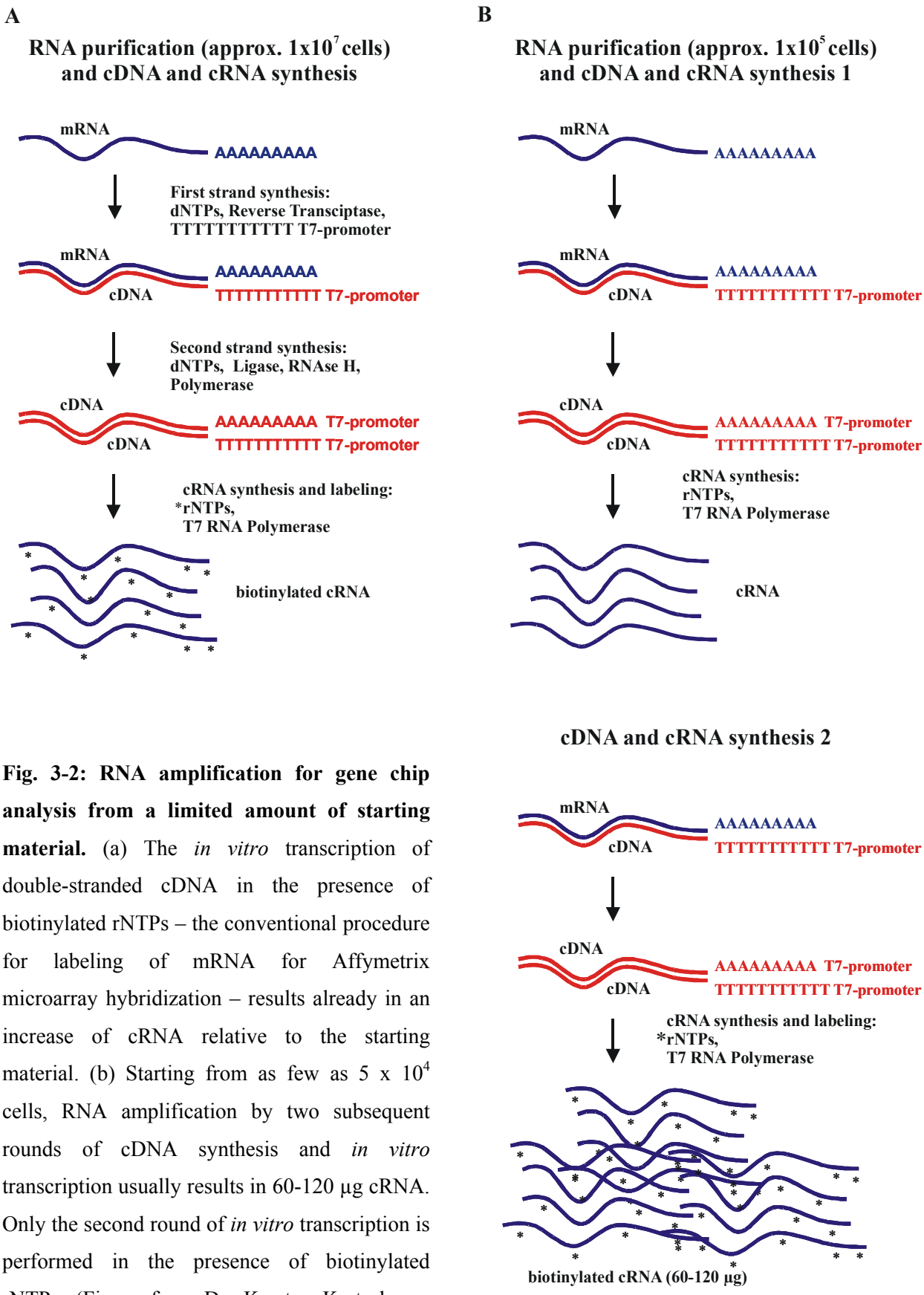


Fig. 3-2: RNA amplification for gene chip analysis from a limited amount of starting material. (a) The *in vitro* transcription of double-stranded cDNA in the presence of biotinylated rNTPs – the conventional procedure for labeling of mRNA for Affymetrix microarray hybridization – results already in an increase of cRNA relative to the starting material. (b) Starting from as few as 5 x 10^4 cells, RNA amplification by two subsequent rounds of cDNA synthesis and *in vitro* transcription usually results in 60-120 µg cRNA. Only the second round of *in vitro* transcription is performed in the presence of biotinylated rNTPs. (Figure from Dr. Karsten Kretschmer, dissertation, TU Braunschweig 2003)

12.5 µg biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MG-U74Av2 chips for 16 hours. After hybridization, GeneChips were washed, stained with streptavidine-PE and read using an Affymetrix GeneChip fluidic station scanner. Scanned raw data images were processed with Affymetrix GeneChip software. For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray suite (Affymetrix). The 16 pairs of oligonucleotides (probe pairs) representing every gene or EST in a probe set, with one sequence being complementary to the target sequence and the other having a 1 bp mismatch in a central position were used as an internal control for hybridization specificity. The relative abundance is reported as the averaged difference of the fluorescence intensity values between the perfectly matched and the mismatched oligonucleotides, resulting in the so-called 'Average-Difference' value (Lockhart *et al.*, 1996; Wodicka *et al.*, 1997).

Probability of differential expression was calculated with unpaired *t*-test statistics (Data Mining Tool, Affymetrix). Genes were considered differentially expressed if the *t*-test results in a confidence level of at least 99%, and if they changed at least twofold with a difference in mean average difference value of at least 100.

3.2. Cell culture

3.2.1. Culture conditions

Embryonic feeder (EF) cells and embryonic stem (ES) cells were cultivated in their appropriate culture medium at 37°C and 7.5% CO_2 in a humidified atmosphere.

3.2.2. Culture of embryonic feeder cells

To maintain their pluripotency, ES cells are usually cultured either on gelatinized plastic plates in the presence of leukemia-inhibitory factor (LIF) or on a layer of mitotically inactive feeder cells derived from embryonic fibroblasts. In this work the ES cells were cultured both on mitomycin C-treated primary EF cells and in the presence of LIF.

Preparation and culture of EF cells obviously needs to be done in advance of any ES cell work and it should be planned, how many feeder cells are required for the experiments. It is

possible to passage and expand EF cells 3 to 4 times, until they will age and lose their capacity to divide. The distinct passages were numbered starting with EF_1 after the first thawing of the primary feeders. Normally only feeder cells until EF_3 were used, and only for short culture periods. For homologous recombination experiments solely EF_1 and EF_2 were used.

A tube of primary feeder cells was thawed and distributed to two or three 15 cm plates. Once being confluent, they were split into three plates and further expanded. When required, confluent plates were treated with mitomycin C (mmc) to inhibit proliferation. After this inactivation, the EF cells were distributed after counting to the required plates (Table 3-1). These plates were gelatinized in the case of a long-time cultivation (e.g. the selection for homologous recombinants). For gelatinization the fresh plates were covered with the gelatin solution for at least 5 minutes at 37°C. Directly before adding medium, the gelatin was removed.

Gelatin: 2% solution, Type B: From Bovine Skin (SIGMA) diluted 1:20 in PBS.

PBS: Dulbecco's phosphate buffered saline (PAA Laboratories GmbH)

Table 3-1: Numbers of EF cells required for the different plates.

Plate	Total surface area (cm^2)	EF cells per plate
15 cm	148	not applicable
10 cm	55	3×10^6
6 cm	21	1.2×10^6
6 well plate	57	3×10^6
12 well plate	45.6	2.5×10^6
24 well plate	45.6	2.5×10^6
48 well plate	38.4	2.1×10^6
96 well plate	30.7	1.7×10^6

3.2.3. Trypsinization

Medium was removed from the culture and the cells were washed twice with PBS. Then Trypsin/EDTA was added and the plates were incubated for 5 minutes at 37°C. After

detaching and isolating of the cells, the reaction was stopped by the addition of medium. The cells were spun down (10 min, 300 x g, 4°C) and plated on new plates.

Trypsin: Trypsin-EDTA (Gibco/Invitrogen) diluted 1:10 in PBS.

3.2.4. Mytomycin C (mmc) treatment

The medium from a confluent plate of EF cells was replaced by mmc-medium. The culture was incubated for two to four hours at 37°C. After this incubation the cells were washed three times with PBS and were then trypsinized and distributed to the desired plates.

mmc-medium: 2 g Mytomycin c (SIGMA) were dissolved first in 2 ml medium and then further diluted in 200 ml medium. After filter sterilization, 10 ml aliquots were stored at –20°C.

3.2.5. Culture of embryonic stem (ES) cells

The ES cells were always cultivated on a layer of mitotically inactive feeder cells. Only cells used for DNA preparation were cultured on gelatinized plates.

The primary goal in handling ES cells was to preserve their pluripotency with respect to embryonic development. ES cells were treated as carefully as possible, thus the medium was changed every day. The cell density was also very important – cultures should be split frequently (every 2 – 3 days) such that individual colonies do not become too large (< 1000 cells). Similarly, when trypsinizing ES cells it was important to fully resuspend the cell clumps (by gentle pipetting) such that no large aggregates were transferred to the new plates. The reason for these precautions was that large colonies easily differentiate and likely lose their omnipotency. Also, the surface area of the tissue culture plate covered by colonies should not exceed ~ 50% since ES cultures will completely differentiate into endoderm-like cells within a few days if the culture has ever become confluent.

Normal culture of ES cells was performed in 10 cm plates. After a transfection the ES cells had to be expanded from 96 well plates. This was carried out by the use of 48 well plates, 12 well plates and 6 well plates.

3.2.6. Cryo-Conservation of mammalian cells

Cells were trypsinized and spun down. After that the cell pellet was resuspended in a small volume (depending on the cell number) of ice-cold freezing medium and transferred to cryotubes (Nunc). The cells were frozen at -80°C and later stored in liquid N_2.

Freezing medium: 10% Dimethylsulfoxid (DMSO, Merck) in the appropriate FCS.

3.2.7. Thawing of cells

The DMSO from the freezing medium was toxic for the cells. Therefore the thawing of the cells from liquid N_2 was done as quickly as possible by agitating the tube in 37°C water bath. The tube was afterwards cleaned with alcohol before opening. The cells were immediately added to ~ 10 ml medium and spun down. Removal of the supernatant also removes the DMSO. The cells were resuspended in new medium and plated out.

3.2.8. Transfection

ES cells used for electroporation should be growing exponentially since the transfection efficiency and perhaps also the frequency at which homologous recombination occurs appears to be increased in optimally growing cells compared to dense ES cultures. For the same reason it was recommended to feed ES cultures with fresh medium a few hours prior to transfection.

A tube with ES cells was thawed four days before transfection. After two days the cells were split and plated exceptionally at higher density than normal on two to three plates. Prior to transfection the construct must be prepared and linearized. Before the day of transfection, 40 µg of the construct (for every 10^7 cells to be transfected) were linearized by restriction digestion followed by phenol/chloroform extraction and precipitation. The pellet should stay in 70% ethanol over night.

On the day of transfection the ES cells were fed four hours before transfection with fresh medium. The ethanol was removed from the DNA pellet and the DNA was air dried in the open tube in a laminar flow hood. Afterwards, the DNA was resuspended in 400 µl of transfection buffer.

The ES cells were then trypsinized and counted. 10^7 cells were taken per transfection. Excess cells were frozen. The cells for the transfection were spun down and resuspended in 400 µl of transfection buffer. The cells were afterwards mixed with the diluted DNA and transferred to a cuvette. It was electroporated at 240 V in cuvettes with 4 mm electrode distance and 1 cm width, using an electroporator with 500 µF capacitance. After electroporation the cells were incubated 10 minutes at room temperature. The electroporated cells were then transferred into medium and plated onto five 10 cm plates.

The selection was started 48 hours after transfection. ES cell medium with G418 (SIGMA, end concentration 300 µg/ml) was used to feed the cells. 9 to 10 days after transfection all non-transfected cells had died and the surviving resistant clones were ready for isolation.

Transfection buffer: RPMI 1640 w/o L-glutamine, w/o phenol red (Gibco/Invitrogen)

3.2.9. Picking of ES cell colonies

Once selection was complete, resistant clones had to be isolated. Colonies at this point should be fairly large in size (~4000 cells) but still maintain a discrete border or edge. Colonies may even have a dark 'cap' indicative of a high density of cells at the top of the colony. Colonies which had begun to differentiate (and spread out) around the edges ("fried egg") had to be avoided.

One day before picking, sufficient 96 well plates with mmc-treated EF cells were prepared. Four hours before picking, the ES cells were fed with fresh medium. 96 well plates (round bottom) with 50 µl trypsin were prepared for the picked colonies and stored at 4°C until picking. The picked colonies were initially transferred into these plates. After trypsinization, separated cells from the picked colonies were subsequently transferred into the plates with the EF cells. Round bottom plates were used for the trypsinization step, because it was easier to quantitatively remove the isolated ES cells from these plates after treatment.

For picking the ES cells were washed once with PBS and covered then with 12 ml cold PBS. With a dissecting microscope in a laminar flow hood (with outward pressure) and a Gilson pipette, single colonies were gently dislodged from the feeder layer, withdrawn in 20 µl of PBS and transferred immediately to the 96 well plate with trypsin. After 30 minutes of picking, the plate was incubated for 3 to 5 minutes at 37°C for the separation of the cells. The trypsinization was then stopped by the addition of 80 µl of medium. Cells were further

suspended by pipetting up and down and distributed into three 96 well plates that contained feeder cells. To dilute the trypsin, the wells were filled completely with medium.

Two of these three plates were used for the conservation of the picked clones. The third plate was used for DNA preparation and analysis of the clones. The cells of all three plates were fed every day. On two subsequent days (e.g. day 2 and 3) a plate kept for conservation was frozen.

To this end, the cells were washed twice with PBS, 50 µl of trypsin was added and the plates were incubated 3 to 5 minutes at 37°C. The reaction was stopped by the addition of 50 µl of medium, and the cells were suspended by pipetting up and down. 100 µl of ES-FCS/20% DMSO was added to each well and every well was overlaid with sterile mineral oil (SIGMA). The plates were wrapped with parafilm and stored at –80°C.

Cells of the third plate, intended for DNA preparation, were trysinized on day 3 and distributed into three gelatinized 96 well plates, thus expanding now three plates for DNA preparation. The ES cells were grown in these plates till full confluence. Then the cells were washed twice with PBS, and the plates were stored at –20°C after wrapping with parafilm.

3.2.10. Flp-mediated deletion of the selection marker in ES cells

Using a *FRT* flanked neomycin resistance cassette in the targeting construct makes it possible to remove the selection marker, after homologous recombinants have been identified. To delete the *FRT* flanked sequences *in vitro*, the Flp recombinase is transiently expressed in ES cells and clones which have lost the neomycin gene by Flp-mediated, *FRT*-specific recombination were isolated on the basis of G418 sensitivity.

For the deletion 10^7 homologous recombinant ES cells were transfected with 30 µg of supercoiled Flp-e expression vector (for general strategy, see above). After transfection only 1000 cells per 10 cm plate were seeded, the rest was frozen for a possible subsequent plating and picking. After 7 days clones were picked (for general strategy, see above) and distributed into three 96 well plates with mmc-treated EF cells (once ½ and twice ¼ of the cells). The two plates with ¼ of the cells were cultured in medium without G418. In the other plate the ES cells were tested for G418 sensitivity with medium that contained the threefold G418 concentration compared to normal. Three days after picking G418 sensitivity became visible in these plates, because such cells did not grow. The sensitive clones were then expanded from the plates without G418 and were later tested by Southern blot analysis for the deletion.

During expansion, an aliquot of these cells was again tested with medium containing G418 for G418 sensitivity.

3.2.11. Cre transduction

The recombination competence of the *loxP* sites was tested in an *in vitro* assay with isolated His-TAT-NLS-Cre protein (Peitz *et al.*, 2002), that can be used to treat cells directly. In this assay homologous recombinant ES cells were plated on 6 well plates without EF cells (2 x 10^5 cells per well). After 5 hours the medium was removed and replaced by the Cre containing medium with different Cre concentrations (2 µM, 1 µM, 0.5 µM, 0.25 µM, 0.125 µM) for 16 hours. Afterwards the Cre medium was removed and the cells were cultured in normal medium. Four days later the DNA was isolated from the cells and tested by Southern blot analysis.

Cre containing medium: The 180 µM Cre stock was diluted in a 1:1 mixture of DMEM and PBS containing 0.1% pluronic F-68 (SIGMA) and sterilized by filtration.

3.2.12. Preparation of ES cells for injection

Five days before injection a tube with recombinant ES cells was thawed. After two days the cells were split and 1/4, 1/8, 1/16, 1/32 and 1/64 of the cells were plated on fresh EF cells. Three days later the cells from the appropriate plate with the optimal appearance were prepared for the injection.

The cells were trypsinized and spun down. After resuspension in 10 ml medium they were plated on a gelatinized 10 cm plate. After 30 minutes the EF cells have attached to the plastic while the ES cells are still in the supernatant or are loosely attached at the bottom. To separate the ES cells from the EF cells the supernatant was transferred to a fresh tube (tube 1). Then the loosely attached cells are rinsed with 10 ml fresh medium from the plate and transferred to another tube (tube 2). The cells are then spun down, resuspended in 500 µl of injection medium and used afterwards for injection.

Blastocyst medium:	500 ml	Dulbecco's MEM with Glutamax-1 (Gibco/Invitrogen)
	90 ml	Foetal bovine serum (Gibco/Invitrogen)
	6 ml	Sodium pyruvate MEM 100MM (Gibco/Invitrogen)
	6 ml	NEAS (Gibco/Invitrogen)
	6 ml	Penicillin-Streptomycin (Gibco/Invitrogen)
	6 ml	L-Glutamine 200MM (Gibco/Invitrogen)
	16.7 ml	HEPES (Gibco/Invitrogen)

Injection medium:	50 ml	Blystocyst medium
	7.5 mg	DNase (SIGMA)

3.3. Immunological methods

3.3.1. Cell sorting

Distinct cell populations can be distinguished on the basis of their differential expression of cell-surface proteins. These cell-surface proteins can be detected using specific antibodies conjugated with fluorescent dyes and analyzed with a flow cytometer or a fluorescence-activated cell sorter (FACS). Besides the bound fluorescence marker, also size and granularity of the cells can be determined.

Single cell suspensions of bone marrow cells were obtained by flushing femur and tibia with FACS buffer. Alternatively, single cell suspensions from fetal livers were obtained using a 100 µm-cell strainer (Falcon). After lysis of erythrocytes the cells were stained with the appropriate antibodies for 15 minutes at 4°C in the dark. After washing twice, the biotinylated antibody was revealed by 10 minutes incubation with Streptavidin-R-PE. After subsequent washing cell sorting was performed on a MOFLO cell sorter (Cytomation). A total of 100.000 cells were sorted directly in the RLT buffer of the RNeasy kit (QIAGEN). Sorted cells were reanalyzed and were 95-99% pure. For the sorting experiment bone marrow of three mice and fetal liver of three to five fetuses were pooled.

1x PBS:	8 g NaCl
	0.2 g KCl
	1.44 g Na_2HPO_4 x 2 H_2O
	0.2 g KH_2PO_4
	dissolved in 1 liter H_2O (pH 7.0) and autoclaved.
FACS buffer:	1x PBS
	2% FCS
	2 mM EDTA
ACK lysis buffer:	150 mM NH_4Cl
	10 mM $KHCO_3$
	0.1 mM Na_2EDTA
	pH 7.2-7.4, filter sterilized (0.2 µm).

4. Results

Many aspects in B cell development, especially the precise differences in fetal liver and adult bone marrow, are largely unknown. In the present work, first germline transcripts of both immunoglobulin light chain loci were cloned and characterized, thus obtaining the prerequisite for subsequent experiments. In these experiments, differential expression of germline transcripts of V gene segments was investigated. The results were then compared with the appearance of JC germline transcripts during B cell development.

Furthermore, a comparison of gene expression patterns between B cell precursors derived from fetal liver and adult bone marrow was performed, using microarrays. Thus, an extensive information about differentially expressed genes was obtained. Finally, a new mouse model with inducible lymphopoiesis was established that should allow to investigate the potential of B cell precursors that arise in neonatal and adult bone marrow.

4.1. Analysis of germline transcripts of immunoglobulin light chain variable regions

In order to study the appearance of transcripts of particular V region families, germline transcripts of V gene segments from both light chain loci had to be isolated and characterized first (Dr. Holger Engel, unpublished and Sandra Düber, diploma thesis, TU Braunschweig, 2001). For this isolation and the subsequent investigation of the expression of germline transcripts of V and JC clusters, the synchronously differentiating pro-B cell line R2-bfl was used (Grawunder *et al.*, 1995b). This line had previously been used to establish differential expression of JC germline transcripts of the κ and λ loci (Engel *et al.*, 1999). R2-bfl cells were derived from fetal liver from *Rag2* deficient mice. The mice were also transgenic for bcl-2, an anti-apoptotic gene, which improves the survival of cells in culture. When grown on bone marrow stromal cells in the presence of IL-7, R2-bfl cells can be kept in the pro-B cell stage. Upon removal of IL-7, these cells almost synchronously differentiate to the phenotype of mature B cells (Grawunder *et al.*, 1995b). Due to the *Rag2* gene deficiency, all Ig loci in this cell line remain in germline configuration. Hence, all Ig transcripts in such cells are transcripts of germline gene segments.

The previously cloned germline transcripts of both light chain variable regions were now characterized with regard to their 3'-UTR. The obtained sequence information was then used

to design primers specific for V gene segments. Subsequently the appearance of germline transcripts of V_κ region families and of the V_λ gene segments was determined in differentiating R2-bfl cells by semi-quantitative RT-PCR. Finally, the findings were compared to the appearance of JC germline transcripts of both light chain loci during the differentiation process.

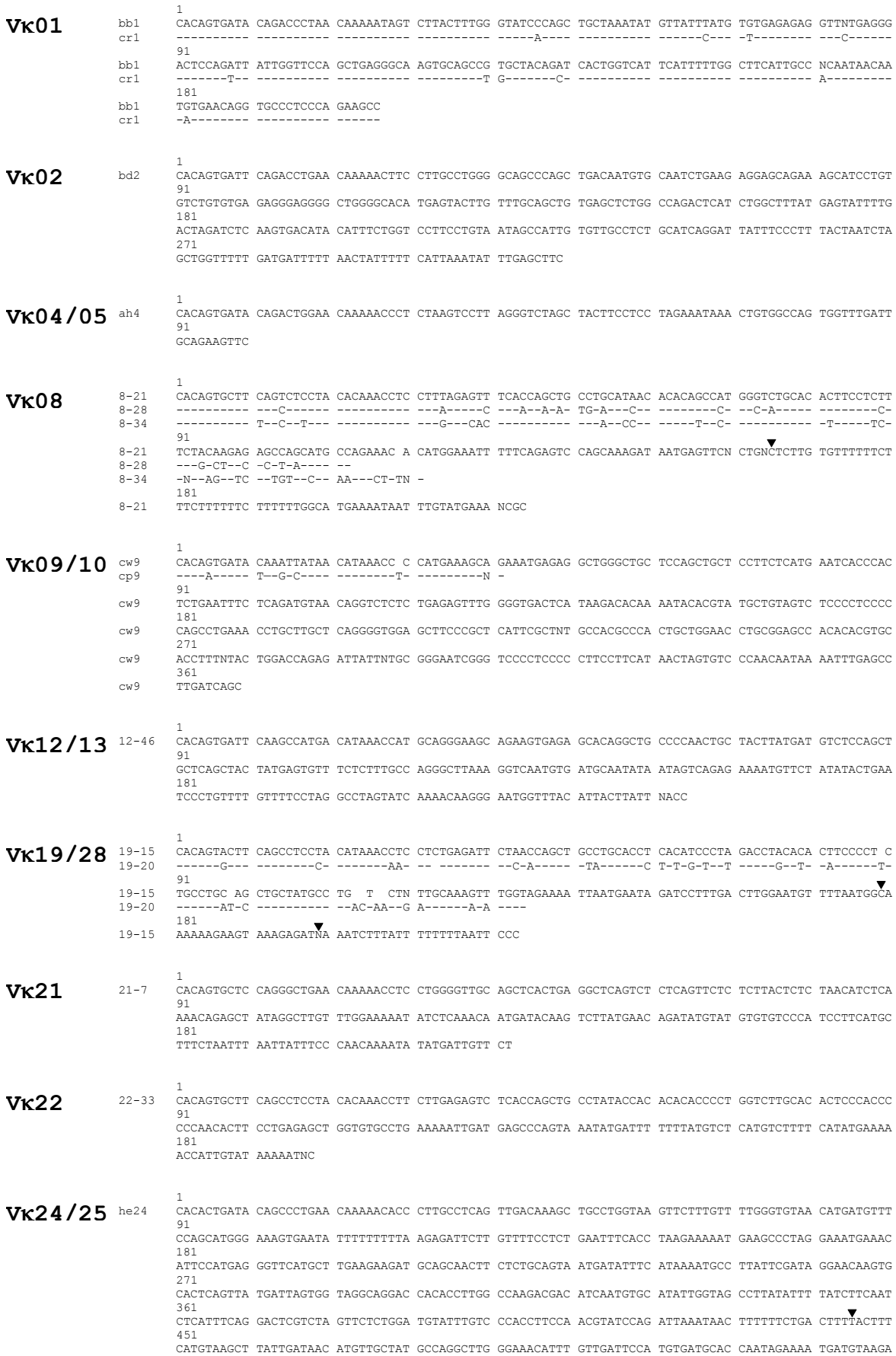
4.1.1. Cloning and characterization of germline transcripts from light chain variable regions

To characterize germline transcripts from V_κ gene segments, *in vitro* differentiation of R2-bfl was allowed for 4 days. At that time the JC clusters of both light chain loci had been shown to be fully activated in these cells and consequently all V regions should be active as well. RNA was isolated (kind gift of Dr. Holger Engel) and used in a V_L-specific 3'-RACE (rapid amplification of cDNA ends). Forward primers for the 3'-RACE were specific for particular members of V_κ families. To distinguish specific from non-specific amplification products, that were observed in some cases, a nested PCR was performed using primers specific for frame work region I of the particular V_κ family. Alternatively, Southern blot analysis was carried out with an oligonucleotide probe that should allow detection of all V_κ regions.

This strategy made it possible to obtain amplification products of germline transcripts for at least one member of 12 of the 18 V_κ families (Fig. 4-1a and Table 4-1). All these transcripts belonged to functional or potentially functional V gene segments. From Fig. 4-1a and Table 4-1 it is also apparent that the 3'-UTR of V_κ gene segments, with few exceptions, are extremely heterogeneous in length and in sequence. In addition, for some germline transcripts alternative polyadenylation sites were found.

Germline transcripts of the three V_λ gene segments were obtained by a similar 3'-RACE. Forward primers for $V_\lambda 1/2$ and $V_\lambda x$ that hybridized to the leader region were designed and amplification products were cloned and characterized. As shown in Fig. 4-1b and Table 4-2, heterogeneity in length and sequence was again found, especially when comparing $V_\lambda 1/2$ and $V_\lambda x$. Even in the case of the $V_\lambda 1$ and $V_\lambda 2$ gene segments, which exhibit extremely high sequence homology at the 3'-UTR, small differences in length were observed. In addition, two distinct polyadenylation sites were found for the $V_\lambda 1$ germline transcripts, whereas for $V_\lambda 2$ only a single site could be detected.

(a)


Vκ01
bb1
cr1
Vκ02
bd2
Vκ04/05
ah4
Vκ08
8-21
8-28
8-34
Vκ09/10
cw9
cp9
Vκ12/13
12-46
Vκ19/28
19-15
19-20
Vκ21
21-7
Vκ22
22-33
Vκ24/25
he24

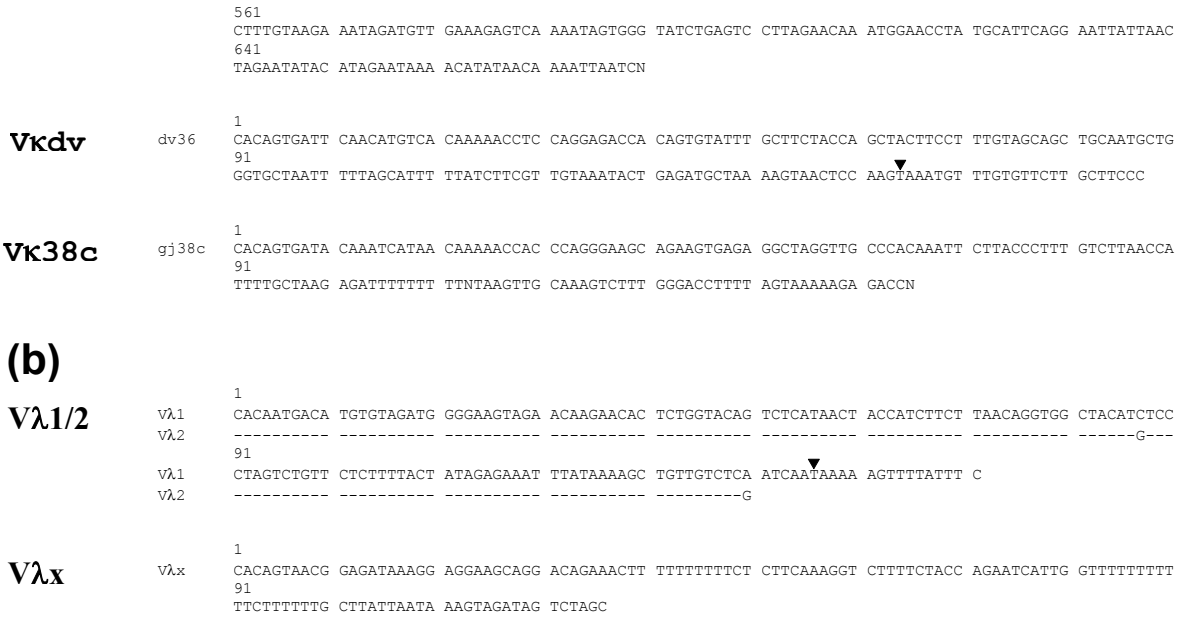


Fig. 4-1: 3'-UTRs of germline transcripts of V_L gene segments of both the κ (a) and the λ (b) locus were analyzed by 3'-RACE. Amplification products were cloned and sequenced. The displayed sequences start at the first nucleotide of the heptamer of the recombination signal sequence. In cases where more than one member of a $V_κ$ family could be analyzed, a sequence comparison was carried out. In this case, identical nucleotides were indicated as dashes for the second and third gene of a family. Arrowheads mark the end of a 3'-UTR due to alternative polyadenylation sites. $V_κ$ genes were named according to the nomenclature described previously (Kirschbaum *et al.*, 1998; Kirschbaum *et al.*, 1999; Roschenthaler *et al.*, 1999; Roschenthaler *et al.*, 2000; Schable *et al.*, 1999; Thiebe *et al.*, 1999).

4.1.2. V region transcripts of light chain loci are differentially activated

The sequence information obtained from germline transcripts of V regions of both light chain loci was combined with previous information on germline transcription of JC clusters. Thus, it was now possible to test, by semi-quantitative RT-PCR, whether germline transcription of the V and JC clusters of a particular light chain locus is initiated at the same developmental stage. In addition, it was investigated whether all V gene segments of the $V_κ$ cluster were activated at the same time, or whether differential activation could be observed. As a first attempt activation kinetics of the two $V_κ$ families that are furthest apart from each other were established. Primers specific for members of the $V_κ02$ family representing the $V_κ$ family most

distal of the JC cluster, and for members of the most proximal V_κ family V_κ21 were employed. Differential activation should be revealed by a delay in the appearance of specific RT-PCR products for either V_κ family in differentiating R2-bfl cells.

Table 4-1: Length of 3'-UTRs from germline V_κ gene transcripts.

Vκ family	Gene	Length of 3'-UTR (bp)	Length of intron[a] (bp)	GenBank Acc. No.[b]
Vκ01	bb1	206[c]	382	AJ231201
	cr1[d,e]	206[c]	377	AJ231205
Vκ02	bd2[f]	319	402	AJ231196
Vκ04/05	ah4[d,e]	100	176	AJ231216
Vκ08	8-21[d]	164 and 223[c]	196	Y15982
	8-28[d]	112[c]	204	AJ235947
	8-34[d,e]	121[c]	202	AJ235958
Vκ09/10	cp9[d]	41	124	AJ231247
	cw9[d]	368	123	AJ231248
Vκ12/13	12-46[d]	244	123	AJ235956
Vκ19/28	19-15[d]	173, 193 and 217	177	Y15976
	19-20[d]	133	292	Y15981
Vκ21	21-7	222[c]	241	K02158/Y15970
Vκ22	22-33[d]	198	203	AJ235965
Vκ24/25	he24	445 and 670	358	AJ132683
Vκdv	dv36[d,e]	153 and 177	172	AJ235966
Vκ38c	gj38c[d]	155	121	AJ235935

[a] For comparison, the length of the intron between exons 1 and 2 is indicated.

[b] Accession numbers of gene sequences described in references (Kirschbaum *et al.*, 1998; Kirschbaum *et al.*, 1999; Roschenthaler *et al.*, 1999; Roschenthaler *et al.*, 2000; Schable *et al.*, 1999; Thiebe *et al.*, 1999).

[c] Generated with a FWR1 specific forward primer.

[d] Transcriptional orientation opposite to the JC_κ cluster.

[e] No expression product, usually cDNA, derived from the germline gene could be found in the database.

[f] The found sequence is most homologous to this gene.

Table 4-2: Length of 3'-UTRs from germline V_λ transcripts.

Gene	Length of 3'-UTR (bp)	Length of intron[a] (bp)	GenBank Acc. No.[b]
Vλ1	146 and 161	90	X58409
Vλ2	140	93	X58423
Vλx	126	104	D38129

[a] For comparison, the length of the intron between exons 1 and 2 is indicated.

[b] Accession numbers of gene sequences described in references (Kirschbaum *et al.*, 1998; Kirschbaum *et al.*, 1999; Roschenthaler *et al.*, 1999; Roschenthaler *et al.*, 2000; Schable *et al.*, 1999; Thiebe *et al.*, 1999).

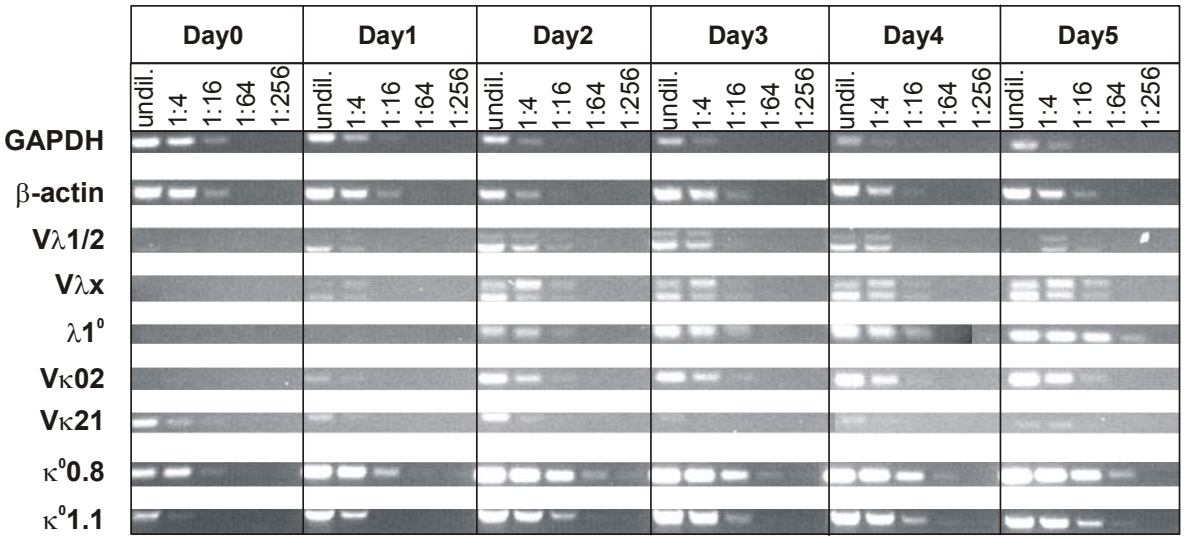


Fig. 4-2: Differential activation of germline transcription at the light chain loci. The expression of germline transcripts of the light chain loci was analyzed in R2-bfl cells by semi-quantitative RT-PCR at various time points after initiation of differentiation by removal of IL-7. Total RNA was isolated and reverse transcribed into cDNA. The cDNA was PCR-amplified undiluted and after serial fourfold dilution with primer pairs specific for germline transcripts of V gene segments (Vλ1/2, Vλx, Vκ02, Vκ21) or JC clusters ($\lambda 1^0$, $\kappa^0$0.8, $\kappa^0$1.1) from both light chain loci. For standardization cDNA primer sets specific for β-actin and GAPDH were used. The additional upper band in the case of Vλ1/2 and Vλx resulted from contaminating genomic DNA indicated by the size of the amplification product. Controls with water showed no product in any case.

To this end, IL-7 was removed from R2-bfl cultures to start synchronous differentiation. Cells were harvested on each consecutive day, RNA was extracted from the aliquots (kind gift of Dr. Holger Engel) and subjected to the semi-quantitative RT-PCR, using the V and JC

specific primers for the κ chain gene segments as well as several primers for housekeeping genes to standardize the amplification. As demonstrated in Fig. 4-2, the two JC_κ germline transcripts κ0.8 and κ1.1 were already detectable at low levels at day 0. The same was true for transcripts of the most proximal V_κ family $V_\kappa 21$. Thus, transcripts of JC_κ and $V_\kappa 21$, as far as can be determined within this system, became apparent at the same stage of differentiation. Interestingly, the appearance of transcripts of the most distal V_κ family $V_\kappa 02$ is delayed compared to transcripts of the $V_\kappa 21$ family.

RNA from the above cells was also used for detection of germline transcripts derived from the λ locus. JC_λ transcripts were weakly detectable first at day 2 after the start of differentiation of R2-bfl, confirming the previous results on differential expression of JC germline transcripts of the two L chain loci. Interestingly, weak expression of both V_λ germline transcripts was apparent already after one day of differentiation. Thus, activation of germline transcription of V_λ and JC_λ clusters is slightly asynchronous. Nevertheless, germline transcription of V_λ and JC_λ starts later than that of JC_κ and the most proximal $V_\kappa 21$ family. However, V_λ and the most distal $V_\kappa 02$ family seem to be activated roughly at the same time.

4.2. Comparison of gene expression patterns between B cell precursors derived from fetal liver and adult bone marrow

The B lymphopoiesis in the fetal liver is in many aspects extremely similar to the B lymphopoiesis in the adult bone marrow. Thus, the basic concept of B cell development, with its correlation between cell surface and intracellular markers and the status of Ig gene rearrangement, is applicable to both hematopoietic sites. However, despite these similarities, two different B cell subpopulations apparently arise from these two distinct anatomical locations as concluded from cell transfer experiments. These experiments led to the hypothesis of two distinct progenitors. Additional support for this idea came from further differences in lymphopoiesis described for fetal and adult life (Douagi *et al.*, 2002; Kincade *et al.*, 2002; Hardy, 2003). Hence, particular molecules are known to be differentially expressed in B cell precursors from either hematopoietic sites, like the myosin light chain (Oltz *et al.*, 1992), TdT (Li *et al.*, 1993) and MHC class II molecules (Lam and Stall, 1994; Hayakawa *et al.*, 1994).

To obtain more complete information about differentially expressed molecules, gene expression analysis using microarrays should be employed. Thus providing further evidence

for distinct progenitors and to identify potential marker genes. The comparison of gene expression was performed on pro- or pre-B-I cells from the liver of 17 days old fetuses or from bone marrow of 7 weeks old BALB/c mice (adult bone marrow). These cells were isolated on the basis of their expression of CD19 and c-kit (Fig. 4-3).

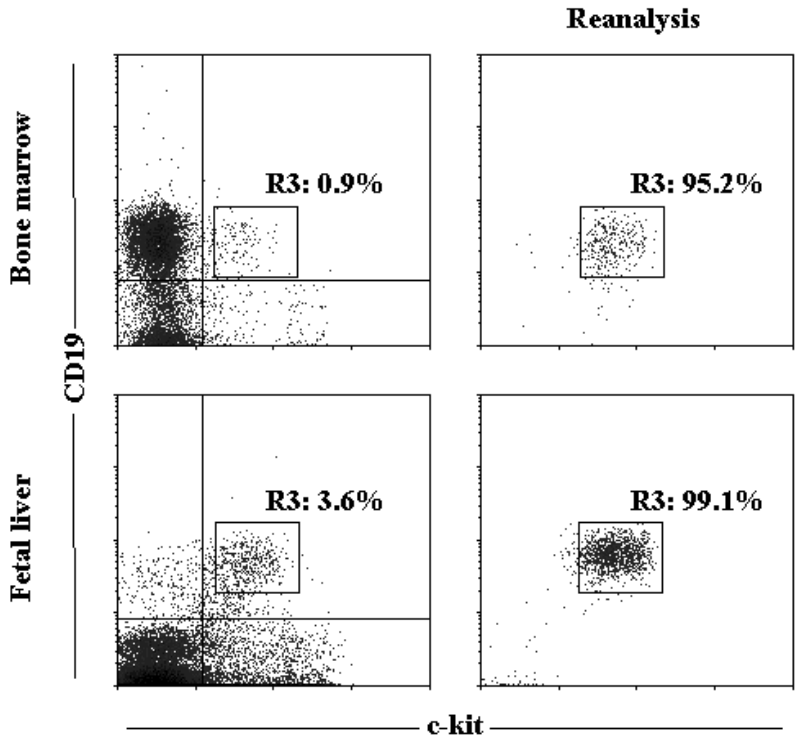


Fig. 4-3: Isolation of B cell precursors. The pro- or pre-B-I cells were FACS purified on the basis of their expression of CD19 and c-kit from fetal liver day 17 or bone marrow of 7 weeks old BALB/c mice. After the sort the isolated cells were reanalyzed to determine the purity of the sorted population.

This developmental stage was chosen for analysis because it represents the first cell stage that is committed to the B lineage. At this stage the Ig loci should be still completely in germline configuration or at the heavy chain locus DJ_H segments might be rearranged. Components of the recombination machinery like the Rag proteins are detectable in these cells (Grawunder *et al.*, 1995a), indicative for the ongoing rearrangement process in such cells.

Total RNA was extracted from 10^5 sorted cells from both anatomical sites and used for mRNA amplification by two subsequent rounds of cDNA synthesis and *in vitro* transcription. The samples were then hybridized to high-density oligonucleotide arrays interrogating approximately 12.000 transcripts (Affymetrix). Three independent replicate experiments were performed and the probability of differential expression was calculated with unpaired *t*-test statistics.

Between B cell precursors from fetal liver or adult bone marrow, a total of 88 genes – 70 known genes and 18 expressed sequence tags (ESTs) - were identified as differentially expressed by a factor of 2 or more with a confidence level of at least 99% (Fig. 4-4).

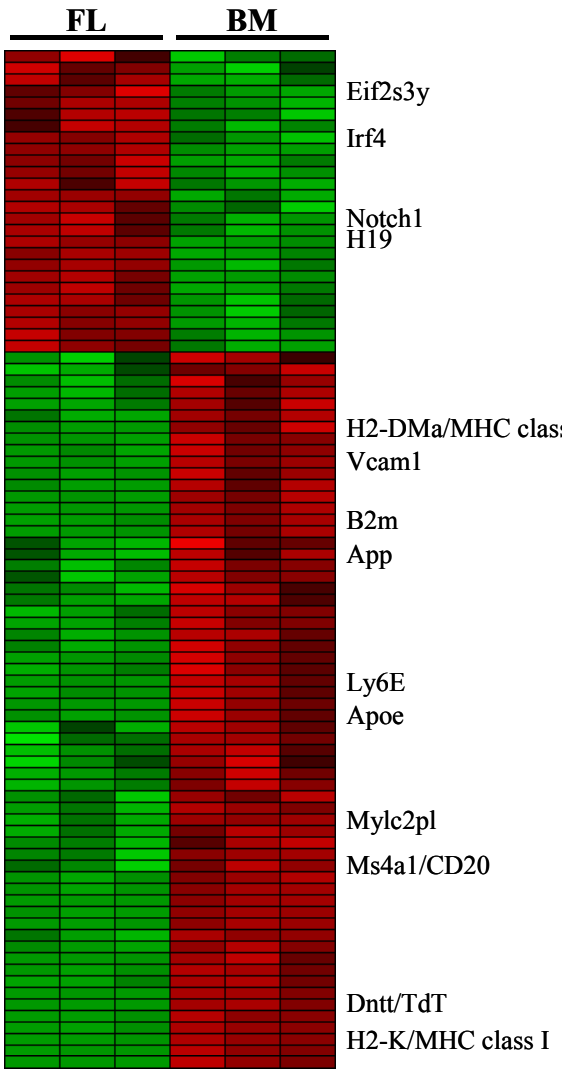


Fig. 4-4: Hierarchical cluster analysis of 88 differentially expressed genes and ESTs as detected by unpaired *t*-test statistics with 99% confidence. Genes or ESTs were considered differentially expressed if they changed at least twofold with a difference in mean average difference value of at least 100. Normalized genes are organized in rows. Green denotes an expression level above, red an expression level below the mean. The columns represent the replicate experiments from fetal liver (FL) or bone marrow (BM), respectively.

By lowering the confidence level, the number of differentially expressed genes can be increased to 133 genes (at 95% confidence). However, this also enhances the possibility of false positive or false negative values. Thus, only the values that were found within the limits of 99% confidence were taken for first analysis (Fig. 4-5).

In fetal liver derived precursor cells, 26 genes were up-regulated compared to bone marrow derived cells. Expression of 14 genes was altered at least 2-fold, 5 were at least 3-fold and 7 at least 5-fold regulated. Eight of these regulated genes were absent or close to background expression levels in the bone marrow population, six of these eight genes belonged to the group of at least 5-fold regulated genes. Five ESTs were up-regulated in the fetal liver. For two of them sequence homology with known proteins could be found in the database.

In the bone marrow derived precursor cells, 62 genes were up-regulated compared to fetal liver derived cells. Expression of 19 genes was altered at least 2-fold, 13 were at least 3-fold, 13 at least 5-fold and 17 at least 10-fold regulated.

Affy_id	Gene Symbol	Description	FL	SD	BM	SD	Fold Change
93028_at	H19	H19 fetal liver mRNA	**760**	32	**42**	21	18.2
103674_f_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3	**198**	32	**17**	11	9.9
102627_at	Igf2bp1	insulin-like growth factor 2, binding protein 1	**245**	20	**25**	15	9.7
160074_at	Ddc	dopa decarboxylase	**386**	40	**44**	23	8.7
95183_i_at	---	EST	**166**	7	**14**	8	8.3
98149_s_at	1110033J19Rik	(similar ribosomal protein)	**581**	25	**70**	14	8.3
95758_at	Scd2	stearoyl-Coenzyme A desaturase 2	**910**	64	**135**	23	6.7
161050_at	---	EST	**515**	38	**117**	49	4.4
162077_f_at	Scd2	stearoyl-Coenzyme A desaturase 2	**1187**	19	**275**	72	4.3
96791_at	1500005K14Rik	(similar hypothetical protein)	**573**	12	**178**	35	3.2
104592_i_at	Mef2c	myocyte enhancer factor 2C	**164**	12	**55**	8	3.0
99327_at	Prss19	protease, serine, 19 (neuropsin)	**172**	5	**57**	16	3.0
97497_at	Notch1	Notch gene homolog 1, (Drosophila)	**521**	51	**181**	25	2.9
93021_at	Rex3	reduced expression 3	**454**	17	**171**	23	2.7
92646_at	Mrpl23	mitochondrial ribosomal protein L23	**1178**	115	**478**	86	2.5
101561_at	Mt2	metallothionein 2	**304**	39	**126**	24	2.4
92737_at	Irf4	interferon regulatory factor 4	**1103**	37	**461**	74	2.4
97450_s_at	Aldh7a1	aldehyde dehydrogenase family 7, member A1	**264**	16	**110**	11	2.4
92796_at	Akp2	alkaline phosphatase 2, liver	**278**	19	**117**	22	2.4
101494_at	Afp	alpha fetoprotein	**218**	17	**94**	10	2.3
161889_f_at	Aldo1	aldolase 1, A isoform	**416**	44	**180**	22	2.3
104716_at	Rbp1	retinol binding protein 1, cellular	**274**	24	**122**	31	2.3
97374_at	2810025M15Rik	EST	**780**	45	**351**	23	2.2
160568_at	Eno1	enolase 1, alpha non-neuron	**1601**	125	**730**	89	2.2
98440_at	Ltb4dh	leukotriene B4 12-hydroxydehydrogenase	**3962**	375	**1835**	161	2.2
96066_s_at	Pkm2	pyruvate kinase, muscle	**569**	31	**280**	22	2.0
100554_at	Pdlim1	PDZ and LIM domain 1 (elfin)	**103**	12	**211**	22	-2.0
103202_at	Gbp3	guanylate nucleotide binding protein 3	**148**	28	**303**	20	-2.1
101681_f_at	---	(similar MHC class I)	**213**	19	**439**	16	-2.1
103562_f_at	---	(mouse endogenous retrovirus truncated gag protein)	**109**	13	**228**	18	-2.1
96657_at	Sat	spermidine/spermine N1-acetyl transferase	**170**	40	**374**	44	-2.2
100828_at	Myla	myosin light chain, alkali, cardiac atria	**106**	12	**238**	12	-2.2
93063_at	App	amyloid beta (A4) precursor protein	**154**	30	**348**	30	-2.3
103501_at	Pura	purine rich element binding protein A	**238**	22	**544**	40	-2.3
160101_at	Hmox1	heme oxygenase (decycling) 1	**256**	33	**595**	67	-2.3
98284_f_at	H2-T18	histocompatibility 2, T region locus 18	**225**	63	**537**	67	-2.4
97398_at	9130022B02Rik	(similar phosphoenolpyruvate carboxykinase)	**192**	15	**464**	32	-2.4
98092_at	Plac8	placenta-specific 8	**315**	58	**759**	44	-2.4
94831_at	Ctsb	cathepsin B	**185**	39	**447**	59	-2.4
96971_f_at	---	(similar Ig kappa light chain)	**67**	19	**169**	8	-2.5
93865_s_at	H2-T10	histocompatibility 2, T region locus 10	**210**	11	**543**	23	-2.6
104680_at	Ramp1	receptor (calcitonin) activity modifying protein 1	**206**	28	**535**	13	-2.6
102797_at	Rsdr1-pending	retinal short-chain dehydrogenase/reductase 1	**84**	13	**220**	15	-2.6
103517_at	---	EST	**63**	11	**168**	12	-2.7
100611_at	Lyzs	lysozyme	**62**	8	**181**	20	-2.9
99379_f_at	H2-K	histocompatibility 2, K region	**185**	45	**583**	14	-3.2
97700_at	---	(similar MHC class I)	**121**	23	**407**	44	-3.4
97349_at	2610019M19Rik	(similar FERM-domain-containing protein)	**71**	4	**241**	16	-3.4
98000_at	Ly64	lymphocyte antigen 64	**64**	34	**241**	23	-3.8
101487_f_at	Ly6e	lymphocyte antigen 6 complex, locus E	**1062**	44	**4040**	390	-3.8
99446_at	Ms4a1	membrane-spanning 4-domains, subfamily A	**67**	28	**263**	19	-3.9
102824_g_at	---	(Ig gamma-2b chain C region, membrane-bound)	**65**	13	**261**	32	-4.0
99071_at	Mpeg1	macrophage expressed gene 1	**68**	6	**271**	31	-4.0
103035_at	Tap1	transporter 1, ATP-binding cassette, sub-family B	**137**	24	**551**	63	-4.0
101876_s_at	H2-T17	histocompatibility 2, T region locus 17	**145**	24	**625**	44	-4.3
102064_at	Casp1	caspase 1	**34**	13	**163**	11	-4.7

103240_f_at	Ear2	eosinophil-associated ribonuclease 2	**66**	19	**314**	25	-4.8
98543_at	Ctss	cathepsin S	**258**	24	**1234**	42	-4.8
100944_at	---	EST	**28**	10	**145**	10	-5.2
93088_at	B2m	beta-2 microglobulin	**1070**	128	**5600**	291	-5.2
102161_f_at	---	(similar MHC class I)	**180**	16	**976**	92	-5.4
101468_at	Pfc	properdin factor, complement	**22**	16	**124**	8	-5.5
93320_at	Cpt1a	carnitine palmitoyltransferase 1, liver	**179**	18	**1142**	63	-6.4
99378_f_at	H2-Q1	histocompatibility 2, Q region locus 1	**82**	15	**570**	71	-7.0
101195_at	Mylc2pl	myosin light chain 2, precursor lymphocyte-specific	**26**	16	**199**	3	-7.7
93092_at	H2-DMa	histocompatibility 2, class II, locus DMa	**25**	3	**192**	23	-7.7
93120_f_at	H2-K	histocompatibility 2, K region	**643**	41	**5010**	217	-7.8
100607_at	Pld3	phospholipase D3	**14**	2	**168**	14	-8.4
160965_at	Rasa4	RAS p21 protein activator 4	**30**	16	**260**	41	-8.8
101753_s_at	Lzp-s	P lysozyme structural	**6**	1	**187**	10	-9.3
97540_f_at	H2-D1	histocompatibility 2, D region locus 1	**624**	118	**6005**	756	-9.6
98438_f_at	H2-Q7	histocompatibility 2, Q region locus 7	**188**	6	**1980**	225	-10.5
92223_at	C1qg	complement component 1, q subcomponent, gamma	**16**	4	**216**	5	-10.8
102904_at	H2-Ea	histocompatibility 2, class II antigen E alpha	**14**	8	**220**	25	-11.0
98562_at	C1qa	complement component 1, q subcomponent, alpha	**41**	14	**468**	27	-11.3
93714_f_at	H2-K	histocompatibility 2, K region	**301**	86	**3779**	136	-12.6
97541_f_at	H2-D1	histocompatibility 2, D region locus 1	**179**	17	**2465**	156	-13.8
96020_at	C1qb	complement component 1, q subcomponent, beta	**34**	24	**480**	49	-14.2
92558_at	Vcam1	vascular cell adhesion molecule 1	**77**	21	**1123**	85	-14.7
101886_f_at	H2-D1	histocompatibility 2, D region locus 1	**171**	1	**2552**	85	-14.9
102823_at	---	(Ig gamma-2b chain C region, membrane-bound)	**10**	9	**330**	42	-16.5
92316_f_at	2010309G21Rik	(Ig lambda light chain)	**26**	4	**424**	35	-16.5
92866_at	H2-Aa	histocompatibility 2, class II antigen A, alpha	**9**	2	**356**	28	-17.8
95356_at	Apoe	apolipoprotein E	**78**	34	**1458**	197	-18.8
102156_f_at	---	(Ig kappa light chain)	**249**	33	**4743**	543	-19.0
103962_at	Dntt	deoxynucleotidyltransferase, terminal	**10**	6	**526**	17	-26.3
161409_f_at	Dntt	deoxynucleotidyltransferase, terminal	**68**	33	**8093**	460	-118.7
103961_s_at	Dntt	deoxynucleotidyltransferase, terminal	**9**	4	**6761**	596	-338.0

Fig. 4-5: Differentially expressed genes between B cell precursors. The unique Affymetrix probe set identifier (Affy_id), the gene symbol with description and the mean average difference value with the standard deviation (SD) of the three replicate experiments from fetal liver (FL) and adult bone marrow (BM) cells are shown. Only genes with a *t*-test confidence level of at least 99% are displayed. ESTs are marked by dashes or the RIKEN cDNA clone number. For complete unknown sequences EST is written in the description. In the case of known protein similarities the possible protein is displayed in brackets. For the calculation of the fold change mean average difference values below 20 were set to 20.

26 of these regulated genes were absent or near background expression levels in the fetal liver population. 17 of these 26 genes belonged to the group of at least 5-fold or 10-fold regulated genes indicating strong differences. 13 ESTs were up-regulated in the bone marrow. For 11 of them sequence homology with known proteins could be found in the database.

Some of the genes up-regulated in the fetal liver derived B cell precursors encoded transcription factors and molecules involved in protein biosynthesis (Mef2c, Irf4, Eif2s3y, 1110033J19Rik and Mrpl23). Of particular interest could be Irf4, a PU.1 interaction partner. Other genes were associated with general metabolism or proteolysis/peptidolysis (Scd2, Aldh7a1, Akp2, Aldo1, Rbp1, Eno1, Pkm2 and Prss19). A gene of the group that was 5-fold or even more regulated, was the dopa decarboxylase (Ddc) with a 8.7-fold higher expression level. The corresponding protein exhibits aromatic-L-amino-acid decarboxylase activity and is involved in the catecholamine biosynthesis. The significance of this however is still unclear.

The gene with the highest up-regulation (18.2-fold) was the H19 fetal liver mRNA. Expression of H19 can be seen in a wide range of embryonic tissues and is down-regulated shortly after birth, except in skeletal muscle. H19 together with the gene for insulin-like growth factor 2 (Igf2) are often held up as a paradigm of genomic imprinting. The H19 and Igf2 genes are thought to be regulated coordinately, both in terms of their common expression pattern and reciprocal imprinting, with H19 being expressed from the maternally inherited allele, whereas Igf2 is paternally transcribed (summarized in Arney, 2003).

The Igf2 gene encodes a major fetal growth factor (Constancia *et al.*, 2002). The function of the H19 mRNA is still unclear. This mRNA contains multiple translation termination signals in all three reading frames. Cellular fractionation has shown that the H19 RNA is found cytoplasmic but not associated with the translational machinery. Thus, it was suggested that the H19 RNA is not a classical mRNA, but may be an RNA molecule that displays a direct function (Brannan *et al.*, 1990). Interestingly also an Igf2 mRNA binding protein (Igf2bp1) was found to be up-regulated in the fetal liver derived precursors. This is consistent with a functional expression of this group of genes.

One further gene with a relatively low up-regulation (2.9-fold) but of particular interest can be found differentially expressed in these precursors, the notch gene homologue 1 (Notch1). Signaling through the Notch cell surface receptor is a highly conserved mechanism of cell fate specification widely used during embryonic development in both vertebrates and invertebrates. When Notch interacts with one of its ligands on the surface of an adjacent cell, the receptor is proteolytically processed, and nuclear translocation of the receptor's intracellular domain results in transcriptional regulation of lineage-specific genes (Weinmaster, 2000; Egan *et al.*, 1998; Artavanis-Tsakonas *et al.*, 1999). Hematopoiesis is also regulated by Notch signaling (Milner and Bigas, 1999). Notch ligands have been

identified in both human (Li *et al.*, 1998) and murine (Jones *et al.*, 1998; Varnum-Finney *et al.*, 1998) bone marrow stroma in addition to murine fetal liver (Walker *et al.*, 2001). Notch receptors have been found in many blood cell types, including developing T cells (Hasserjian *et al.*, 1996; Ellisen *et al.*, 1991) and B cells (Ohishi *et al.*, 2000; Bertrand *et al.*, 2000).

The list of differentially up-regulated genes in bone marrow derived precursors confirmed previous findings. Thus, the myosin light chain (Mylc2pl), TdT (Dntt) and transcripts of the MHC class II cluster (H2-DMa, H2-Ea and H2-Aa) were detectable in these cells whereas they were absent in the fetal liver derived ones. Interestingly, many MHC class I genes were found differentially expressed (H2-T18, H2-T10, H2-K, H2-T17, H2-Q1, H2-D1 and H2-Q7), i.e. low expression levels were found in fetal liver. Class I gene products are heterodimers consisting of a heavy chain that is associated non-covalently with the β2-microglobulin light chain. This β2-microglobulin light chain was also found to be differentially expressed (B2m). In addition, consistent with the above data, cathepsin S (Ctss) that is involved in maturation of MHC class II molecules (Hsieh *et al.*, 2002) and the transporter 1 (Tap1) that is involved in MHC class I presentation (Garbi *et al.*, 2003; Chefalo *et al.*, 2003) were up-regulated in bone marrow derived precursors.

Similar to the fetal liver, some of the genes up-regulated in the bone marrow were transcription factors and molecules involved in nucleotide metabolism (Pdlim1, Sat and Pura). Also an RNase of the RNase family A was found differentially expressed (Ear2). Other genes, associated with general metabolism or proteolysis/peptidolysis (9130022B02Rik, Rsdr1-pending, Cpt1a, Pld3, Ctsb, Casp1) and two molecules involved in cell signaling (Ramp1, Rasa4) were up-regulated compared to the FL derived precursors. Surprisingly, also κ and λ light chains and an Ig gamma-2b chain were found differentially expressed (102156_f_at, 2010309G21Rik, 102824_g_at).

C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation. All three chains of C1q (C1qg, C1qa, C1qb) were found to be up-regulated in the bone marrow derived precursors. But the function of this molecule at this developmental stage is not clear, since complement components are supposed to be synthesized by macrophages and hepatocytes (Morgan, 2000).

The gene for amyloid beta (A4) precursor protein (App) was also found to be up-regulated in the bone marrow derived precursors. The fold change was only 2.3 times, but this protein could nevertheless be interesting. Notch1, that was found to be up-regulated in the fetal liver, and App are processed by the same γ-secretase. In *in vitro* experiments, competition for the

enzyme activity between both molecules was demonstrated (Lleo *et al.*, 2003). In addition, activation of the Notch signaling pathway induced down-regulation of Presenilin 1 (PS1), which is a critical component of the γ-secretase complex. This suggests that the Notch1 pathway exerts some control on PS1 gene regulation that may ultimately affect App processing (Lleo *et al.*, 2003).

A gene with a high up-regulation (18.8-fold) was Apoe. Apolipoprotein E (ApoE) is a glycosylated protein with multiple biological properties. It was originally described in the context of cholesterol metabolism, but effects of ApoE on innate and acquired immune responses have been demonstrated. Thus, ApoE has the ability to suppress lymphocyte proliferation *in vitro* (Avila *et al.*, 1982). In addition, ApoE can stimulate cultured neutrophils (Terkeltaub *et al.*, 1991). More recent data showed that ApoE deficient mice exhibit impaired immune responses after bacterial challenge with *Listeria monocytogenes* (Roselaar and Daugherty, 1998), and increased susceptibility to endotoxemia induced by lipopolysaccharide (LPS; de Bont *et al.*, 1999).

Another interesting differentially expressed molecule was Vcam1 (CD106). This gene product was almost completely absent in fetal liver derived precursors and 14.7-fold up-regulated in the bone marrow derived cells. Vcam1 mediates adhesion of leukocytes but is also supposed to be a costimulatory molecule for T cells (Burkly *et al.*, 1991). Its expression has been described for bone marrow stromal cells (Miyake *et al.*, 1991), follicular dendritic cells (Brasel *et al.*, 1996), stimulated endothelial cells (Rice and Bevilacqua, 1989), but also for splenic B1a cells (Kretschmer *et al.*, 2003b).

Three additional cell surface molecules were found to be up-regulated in bone marrow derived precursors (Ly64, Ly6E and Ms4a1). For Ly64 a possible regulatory role in the cellular response to IL-3 was suggested (Dougherty *et al.*, 1989). A presumable involvement in adhesion to endothelial cells was also hypothesized (Tang *et al.*, 2002). Ly6E is also known as stem cell antigen 2 (Sca-2) or as thymic shared antigen 1 (TSA-1) and belongs to the Ly-6 family, a group of small cysteine-rich cell surface proteins (Classon and Coverdale, 1994).

Ms4a1 is better known as the cell surface antigen CD20. CD20 is expressed only on B lineage cells but is absent from plasma cells (Tedder and Engel, 1994). It is a member of the CD20/FcεRIβ superfamily of leukocyte surface antigens which also includes the β subunit of the high-affinity receptor for IgE (FcεRIβ) and HTm4. It has been demonstrated that CD20 can exist in a complex with MHC class I and II, and the molecules CD53, CD81 and CD82 (Szollosi *et al.*, 1996). No extracellular ligand for CD20 has been identified so far. Indirect

evidence suggests that CD20 functions as a B cell Ca^{2+} channel subunit (Bubien *et al.*, 1993). An ion channel function would be consistent with reports that CD20 regulates cell cycle progression (Kanzaki *et al.*, 1995) and exists on the cell surface as a homo-oligomer (Bubien *et al.*, 1993).

Taken together, comparison of gene expression patterns between fetal liver and bone marrow derived B cell precursors revealed significant differences between both populations. However, from these results it is not yet possible to conclude whether these populations are derived from different precursors or not. This would require detailed functional analysis of the differentially expressed genes. Therefore it was important to generate a mouse model in which this question could be answered more definitely in a holistic approach.

4.3. Generation of mice with inducible *Rag1* expression

The origin of B1a cells, which are suggested to act as a "first line of defense" against mucosal and systemic pathogens, is still controversially discussed. It is unclear, whether these cells are only generated during fetal and perinatal life, or whether the adult bone marrow could also give rise to such B cells. This question should definitely be answered with a gene-targeted mouse that has initially a block in the development, especially during fetal development. This block should be abrogated later in the adult life to allow B cell ontogeny.

Due to the extraordinary developments in modern molecular biology it is possible to turn genes on and off at discretion. In the mouse, this has been accomplished by using binary systems in which gene expression is dependent on the interaction of two components that results in either transcriptional transactivation or DNA recombination. The number of these binary systems has steadily increased, and the initially established systems, such as the Cre/*loxP* recombination and the TetR (tetracycline repressor) based transactivator system, have been optimized.

4.3.1. The search of an appropriate target gene

First was the question of the appropriate target gene to generate such a mouse. It has been demonstrated that gene defects preventing BCR expression, like defects in V(D)J recombination, lead to an arrest in B cell development. But some of these knock-outs were leaky and led to the accumulation of small numbers of B cells, for instance in mice with the scid mutation (Bosma and Carroll, 1991). Other gene defects concerned genes involved in

BCR signaling, like the knock-out of *mb-1*. This gene encodes Igα, that is required for the transport of the BCR onto the cell surface. Together with Igβ, Igα forms a heterodimer that mediates signal transduction from the BCR (Kim *et al.*, 1993). Since signal strength via the BCR seems to have an influence on the generation of different B cell subpopulations, targeting of a molecule involved in signaling should be avoided. Nevertheless, the *mb-1* gene was already used for the generation of knock-in mice using *loxP* sites in opposite orientation (Pelanda *et al.*, 2002). In these mice the generation of different B cell subpopulations was not studied so far.

A more appropriate target seemed to be one of the recombination activating genes – *Rag1* and *Rag2*. Mice in which one of these genes had been inactivated by homologous recombination (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992) were not able to rearrange their antigen-receptor genes leading to a complete lack of all B and T lymphocytes. This complete block in B cell development was the reason to target one of these genes for the generation of a mouse with inducible B cell or lymphocyte development, respectively. The remaining question was whether *Rag1* or *Rag2* should be the target. It has been demonstrated, that in addition to the transcriptional control of both *Rag* genes, the Rag2 protein levels are regulated during cell cycle (Lin and Desiderio, 1994). This posttranslational control is apparently mediated by modulation of Rag2 protein stability resulting from cell cycle-dependent phosphorylation (Lin and Desiderio, 1993). Furthermore, the expression of *Rag2* seems to have an influence on the half-life of Rag1 protein (Grawunder *et al.*, 1996). Since *Rag1* seems to be less regulated than *Rag2*, and *Rag2* expression obviously influences Rag1 protein levels and not vice versa, *Rag1* appeared to be the appropriate target gene. Of course targeting of *Rag1* will also influence the T cell development. However, this was taken as a welcome side effect. Thus, both cellular mediators in adaptive immunity were made inducible at the same time.

4.3.2. The search for the appropriate inducible system

After the decision to target *Rag1*, there was still the question of the experimental system. Site-specific DNA recombination or a TetR based transactivator system were the two alternatives for the project. Two members of the integrase family of site-specific recombinases, Cre from bacteriophage P1 (Sauer and Henderson, 1989) and Flp from *Saccharomyces cerevisiae* (O'Gorman *et al.*, 1991), are presently used to conditionally control gene expression by site-specific DNA recombination. Cre and Flp recombinases catalyze a conservative recombination event between two 34-bp recognition sites (*loxP* and *FRT*, respectively) that

differ in primary sequence but share secondary structure. Because these recombinases do not require accessory proteins or high-energy cofactors for their activity, they are suitable for use in the foreign milieu of a mouse cell. To this end, Flp has been modified to alter its temperature optimum for improved activity at 37°C – the body temperature of mice (Buchholz *et al.*, 1998).

Dependent on the location of the recognition sites and their orientation, the DNA recombination can delete or invert DNA fragments in *cis* or insert or translocate DNA fragments in *trans*. Thus, when both recognition sites are located on the same chromosome (*cis*) as direct repeats, recombinase expression leads to the deletion of the intervening DNA. If the recognition sites have an opposite orientation, recombinase expression inverts the intervening DNA (summarized in Zheng *et al.*, 2001). The inversion of the DNA is a reversible process and the continuous presence of recombinase in the nucleus results in repeated inversions (flipping) of the *loxP* or *FRT* flanked (so called floxed or flrted, respectively) DNA sequence. Ceasing of the nuclear recombinase activity stops further recombination and stabilizes the floxed or flrted DNA into one of the two possible orientations with equal probability.

Tetracycline-dependent regulatory systems were developed by Gossen and Bujard (Gossen and Bujard, 1992). In these systems, the effector molecule is encoded by a fusion of a gene segment that encodes a VP16 transactivation domain and the tetracycline repressor (TetR) gene from *E. coli*. The fusion protein specifically binds both tetracycline and the 19 bp operator sequences (tetO) of the tet operon in the target transgene which results in controllable transcription. There are two versions of this system. In the original system, the tetracycline-controlled transcativator (tTA) can not bind DNA when the inducer is present (‘tet-off’), whereas in a modified version, the ‘reverse tetA’ (rtTA) binds DNA only when the inducer is present (‘tet-on’; Gossen *et al.*, 1995). The current inducer is doxycycline (Dox) because of its low cost, commercial availability, and because it efficiently activates rtTA and inactivates tTA at doses that are well below cytotoxic levels (Baron and Bujard, 2000; Gossen *et al.*, 1995). A disadvantage of this system for the current project was, that TetR based transactivators permit expression only at a transgenic level. Thus, the target genes are not regulated, as they would be under physiological conditions.

However, physiological regulation appears to be especially important for the expression of the Rag proteins. For instance, a negative effect of transgenic *Rag* expression has been shown in two previous studies. In the first study, *Rag1* and *Rag2* were constitutively expressed under

the control of an early thymocyte-specific promoter leading to defects in mature T cell differentiation antigen expression and in T cell receptor-mediated activation, as well as lymphocyte homing defects (Wayne *et al.*, 1994a; Wayne *et al.*, 1994b). In the second study, ubiquitous expression of both *Rag* genes lead to a block in early B and T lymphopoiesis, in addition, to growth retardation and early death of the mice (Barreto *et al.*, 2001). Because of these indications of the importance that correct regulation of *Rag* expression is necessary, it was decided to realize the project by site-specific DNA recombination thereby retaining the full transcriptional control of the target gene.

The correctness of this decision was confirmed in a very recent report (Shockett *et al.*, 2004). In this study it was tried to obtain mice with inducible *Rag* expression using a tetracycline-based auto-regulatory system for inducible gene expression (Shockett *et al.*, 1995). Only partial reconstitution of lymphocyte populations was found in these mice. This study focused on T cell development, thus B1a cells of the peritoneum were excluded from investigation. However, in the transgenic mice only a low level of B cell reconstitution was observed in the spleen, although normal levels of serum immunoglobulin were found in these mice. This measurement of serum Ig levels indicated that some B cells could accumulate in the absence of induction. Thus, the system was not completely closed.

Therefore, this confirmed that the best choice to realize a mouse with inducible *Rag1* expression was obviously the use of site-specific DNA recombination. The two systems that are currently in use to conditionally control gene expression are the Cre/*loxP* and the Flp/*FRT* system. The Cre/*loxP* recombination system is the most frequently used, as it was the first well-developed system. Initial attempts to develop the Flp/*FRT* system in transgenic mice resulted in either no recombination (Ludwig *et al.*, 1996) or mosaic recombination (Dymecki, 1996). This was due to the decreased enzyme stability at 37°C of both wild-type Flp and an Flp variant (Flp-F70L; Buchholz *et al.*, 1996b). The generation of the thermostable variant Flp-e solved this problem (Buchholz *et al.*, 1998). Thus, Flp-e transgenic mice mediate recombination efficiencies resembling those of Cre (Rodriguez *et al.*, 2000) and can be used now as alternative. In contrast to the Flp/*FRT* system, many effector and target transgenic mouse lines exist already for the Cre/*loxP* system, to which newly generated mouse lines can be crossed. Thus the Flp/*FRT* system should be used in the generation of mice in this project only as supplementary system for the removal of the positive selection marker, whereas the gene activation was attempted by using the Cre/*loxP* system.

Two ways exist for the Cre-mediated gene activation. In the first, a STOP cassette, flanked by *loxP* sites in the same orientation, is introduced between the promoter and the gene of interest. Such a STOP cassette can consist of polyadenylation sequences, false translation initiation codons and splice donor sequences, thus preventing transcription and translation of the target gene. A SV40 tumor antigen was shown to be rendered completely quiescent by using such a STOP cassette (Lakso *et al.*, 1992). The STOP cassette can be removed by Cre-mediated DNA recombination leading to normal transcription of the target gene. This method is often used for inducible transgene expression.

The other possibility, and the possibility used in this work, is the flanking of a target gene by *loxP* sites in opposite orientation. If the target gene is first in an inverted orientation, Cre expression will lead to reversion and thus restoration of the normal transcription unit. The continuous presence of recombinase in nuclei results in the repetitive inversion (flipping) of the floxed DNA sequence. Removal of nuclear recombinase, stops further recombination, stabilizing the floxed DNA into one of the two possible orientations with equal probability. Thus only 50% of the cells will show gene activation.

Since it could not be guaranteed that the insertion of a STOP cassette completely silences a given gene, the inversion of *Rag1*, floxed in opposite orientation, was chosen as the appropriate system. It seemed tolerable under these circumstances that only 50% of the lymphocyte precursors might end up with an active *Rag1* gene. Since lymphocyte ontogeny involves extensive proliferation, this defect should be compensated.

4.3.3. Strategy for the deletion of the neomycin resistance cassette

A positive selection marker, e.g. a neomycin resistance cassette, is routinely used to select for recombinant ES cell clones and is necessarily part of the initial floxed allele. However, the placement of the neomycin resistance gene in introns can interfere with gene expression at the floxed allele. This loss of target gene function was observed if the resistance gene was in the same orientation relative to the transcription of the floxed gene (Meyers *et al.*, 1998; Nagy *et al.*, 1998; Postic *et al.*, 1999; Cao *et al.*, 2001). In addition, loss of target gene function was also found for opposite transcriptional orientation (Wang *et al.*, 1999; Hayhurst *et al.*, 2001; Xu *et al.*, 2001). In some additional cases, it has been determined that cryptic splice sites in the neomycin resistance gene interfere with normal splicing and therefore reduce mRNA levels of the target gene (Meyers *et al.*, 1998; Nagy *et al.*, 1998; Xu *et al.*, 2001). Interference of target gene expression was also observed if the neomycin resistance gene has been placed

in 5'- or 3'-flanking or untranslated regions (Lowe *et al.*, 2001; Matsuda *et al.*, 2001; Rucker, III *et al.*, 2000).

A common solution to this problem, that for instance was used in the initial tissue-specific knock-out experiments, is to use three *loxP* sites, positioned such that the neomycin resistance cassette, as well as the essential region of the gene of interest, is floxed (Gu *et al.*, 1994). A partial Cre-mediated recombination event could then remove the cassette and leave the essential region floxed. This partial deletion is most often achieved *in vitro* in ES cells by transiently introducing a Cre-expression cassette.

The generation of a thermostable variant of the Flp recombinase – Flp-e – provided an alternative to this – sometimes inefficient – partial Cre-mediated recombination. A positive selection marker cassette, flanked by *FRT* sites, can now be removed by Flp-mediated recombination. This recombination has no influence on the floxed target gene and can be carried out both *in vitro* and *in vivo*.

4.3.4. Targeting strategy

The *Rag1* gene is located on mouse chromosome 2 and consists of two exons, whereby the whole coding sequence resides in the second exon. The construction of the targeting vector was carried out by using a 9.6 kb genomic BamHI fragment containing the exon 2 of *Rag1* (Fig. 4-6). The complete vector had a short arm of homology of 1.1 kb and 3.5 kb as the long arm of homology. For the initial silencing of the *Rag1* gene, a region, including the CDS, 3.7 kb in size, was inverted to prevent *Rag1* expression. To achieve inducible *Rag1* expression, this region was flanked by *loxP* sites in opposite orientation. Thus, inactivation can be reversed by Cre expression. The first *loxP* site was located in the intron between exon 1 and 2, whereas the second site was localized in the 3'-untranslated region (Fig. 4-6).

Homologous recombination is a rare event in mammalian cells, and therefore a neomycin resistance cassette was inserted into the construct to provide the possibility to enrich transfectants. This cassette was flanked by *FRT* sites as direct repeats to render the selection marker removable (Fig. 4-6). However, G418 resistance per se only indicates that the cells have taken up and integrated the neomycin resistance gene. To be able to select for cells in which homologous recombination has occurred, the construct contained also a negative selection marker, in this case a DTA cassette (Fig. 4-6).

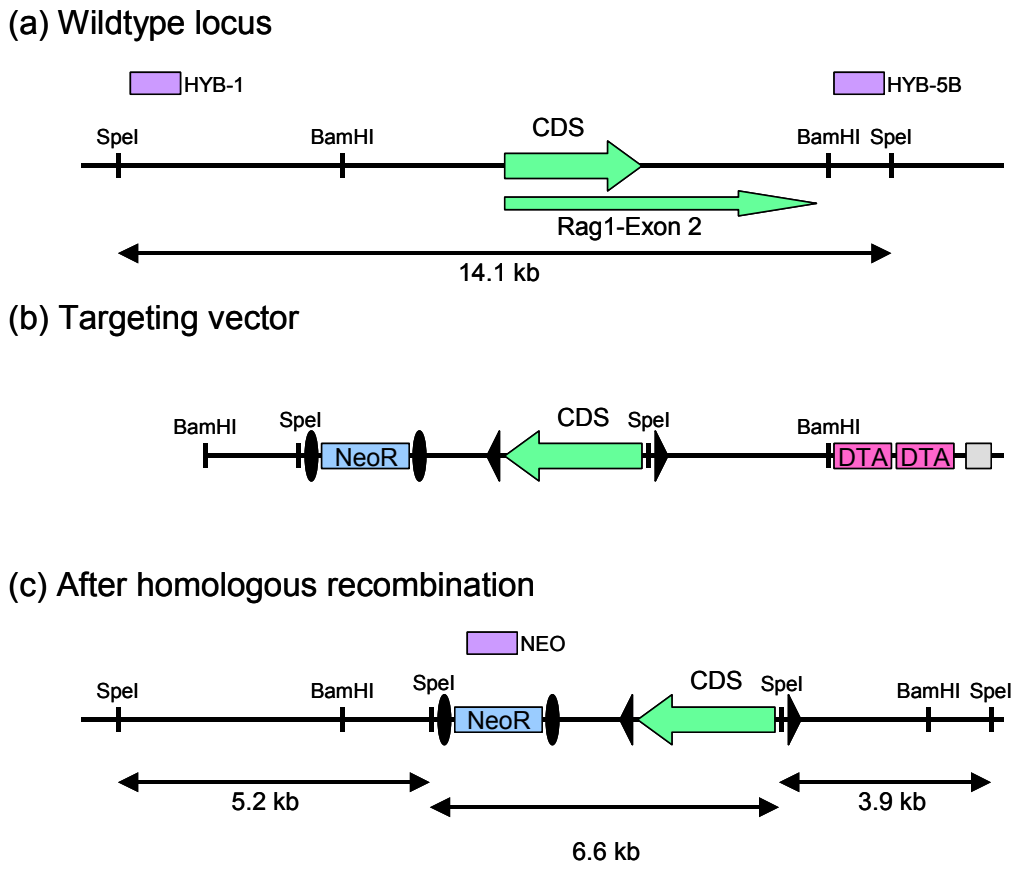


Fig. 4-6: Targeting strategy for the generation of mice with inducible *Rag1* expression. A restriction map of the wild-type Rag1 locus is shown in (a). Indicated are only restriction sites important for the targeting. Probes used to verify targeting events are marked lilac and are shown with the expected size of the restriction fragment after SpeI digestion. The targeting vector is displayed linearized in (b). The BamHI fragment of the Rag1 locus is shown with the vector backbone (gray box). The DTA cassettes are marked red. The complete coding sequence of *Rag1* is now inverted and flanked by *loxP* sites in opposite orientation (indicated by arrowheads). The *FRT* flanked neomycin resistance cassette was inserted into a unique NheI site. Two new SpeI sites were introduced through the cloning. The structure of the targeted Rag1 locus is displayed in (c). The localization of the NEO probe is shown in addition to sizes of diagnostic restriction fragments after SpeI digestion.

This cassette encodes the subunit A of Diphtheria toxin, which ADP-ribosylates elongation factor 2 and thus prevents protein synthesis in all cells that express DTA. Target cells that incorporate DNA randomly usually retain the entire transgenic construct including the DTA cassette and are therefore eliminated. On the other hand homologous recombinants integrate only the sequence between the regions of homology and thus lose the DTA cassette allowing their survival. This results in enrichment of homologous recombinants. For the screening of

homologous recombinants, a strategy based on Southern blot analysis was developed. This strategy included the introduction of two SpeI sites into the targeting vector. Digestion with SpeI and hybridization with the external 5' and 3' probes HYB-1 and HYB-5B results in the 14.1 kb wild-type band (Fig. 4-6a). 'External' indicates here that the probes hybridize outside the region of homology found in the targeting vector. Clones which have integrated the construct by homologous recombination should show, in addition, to the wild-type band a 5.2 kb or a 3.9 kb band, respectively (Fig. 4-6c). It is advisable to choose a restriction fragment size that is smaller than that of the wild-type allele to verify the targeting event, since this excludes false positive results from incompletely digested DNA.

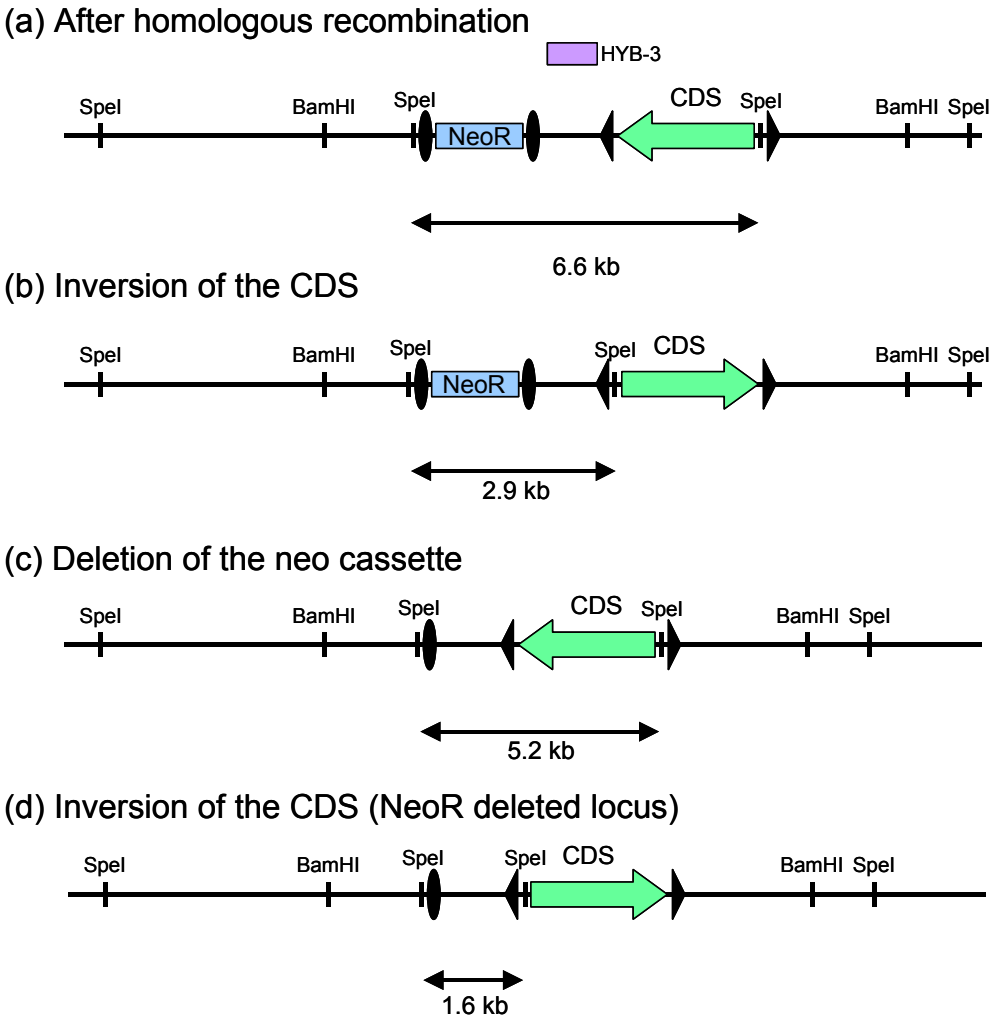


Fig. 4-7: Site-specific DNA recombination. The structure of the targeted Rag1 locus is displayed in (a). The probe used for Southern blot analysis is marked lilac and is shown together with the expected size of the restriction fragment after SpeI digestion. The targeted locus after inversion of the CDS (b), the locus after removal of the neomycin resistance cassette (c) and the previously NeoR deleted locus after inversion of the CDS (d) are shown, including sizes of diagnostic restriction fragments after SpeI digestion.

During establishment of a homologous recombinant it is essential to rule out the possibility that an additional construct has integrated as transgene. Hybridization using a probe specific for the neomycin resistance cassette (NEO; Fig. 4-6c) also needs to be carried out.

In addition to the original homologous recombination, other recombination events, like the inversion of the floxed region or the deletion of the neomycin resistance cassette, can be monitored by Southern blot analysis (Fig. 4-7). To this end, an internal probe can be used. Digestion with SpeI and hybridization with HYB-3 should result in a 2.9 kb band after inversion of the CDS, in addition to the unaltered 6.6 kb fragment. The two bands are found due to the equal probability of both orientations after repeated inversions (Fig. 4-7b).

The final deletion of the neomycin resistance cassette can be detected by appearance of a specific 5.2 kb band when hybridized with HYB-3 (Fig. 4-7c). Similarly, the inversion of the CDS in the locus after the removal of the neomycin resistance cassette can be verified using the same probe by the appearance of a 1.6 kb band (Fig. 4-7d).

4.3.5. Modification of the neomycin resistance cassette

The neomycin resistance cassette was a kind gift of Dr. Werner Müller (Experimental Immunology, GBF Braunschweig). An XhoI/SalI fragment containing the neomycin resistance gene flanked by *FRT* sites was cloned into the vector pBluescript II SK+. To verify the nature of the promoter and to ensure the functionality of the neomycin resistance gene, the whole fragment was sequenced. This revealed that the neomycin resistance gene was under the control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter and contained also the TK polyA signal. The flanking of the resistance cassette by two *FRT* sites in the same orientation was also confirmed this way (Fig. 2-2). With the obtained sequence information it was now possible to modify the neomycin resistance cassette for cloning into the targeting vector (Fig. 4-8).

In a first modification step an NheI site was introduced between the KpnI and the XhoI site using an adapter, leading to NeoR cassette mod1. In a second step, resulting in NeoR cassette mod2, the region between SalI and NotI was removed and replaced by SpeI, XhoI and NheI sites. The SpeI site was important for the screening of recombinants described above. The other modifications made it possible to remove the neomycin resistance cassette either by NheI or XhoI from pBluescript II SK+ and to use it for further cloning.

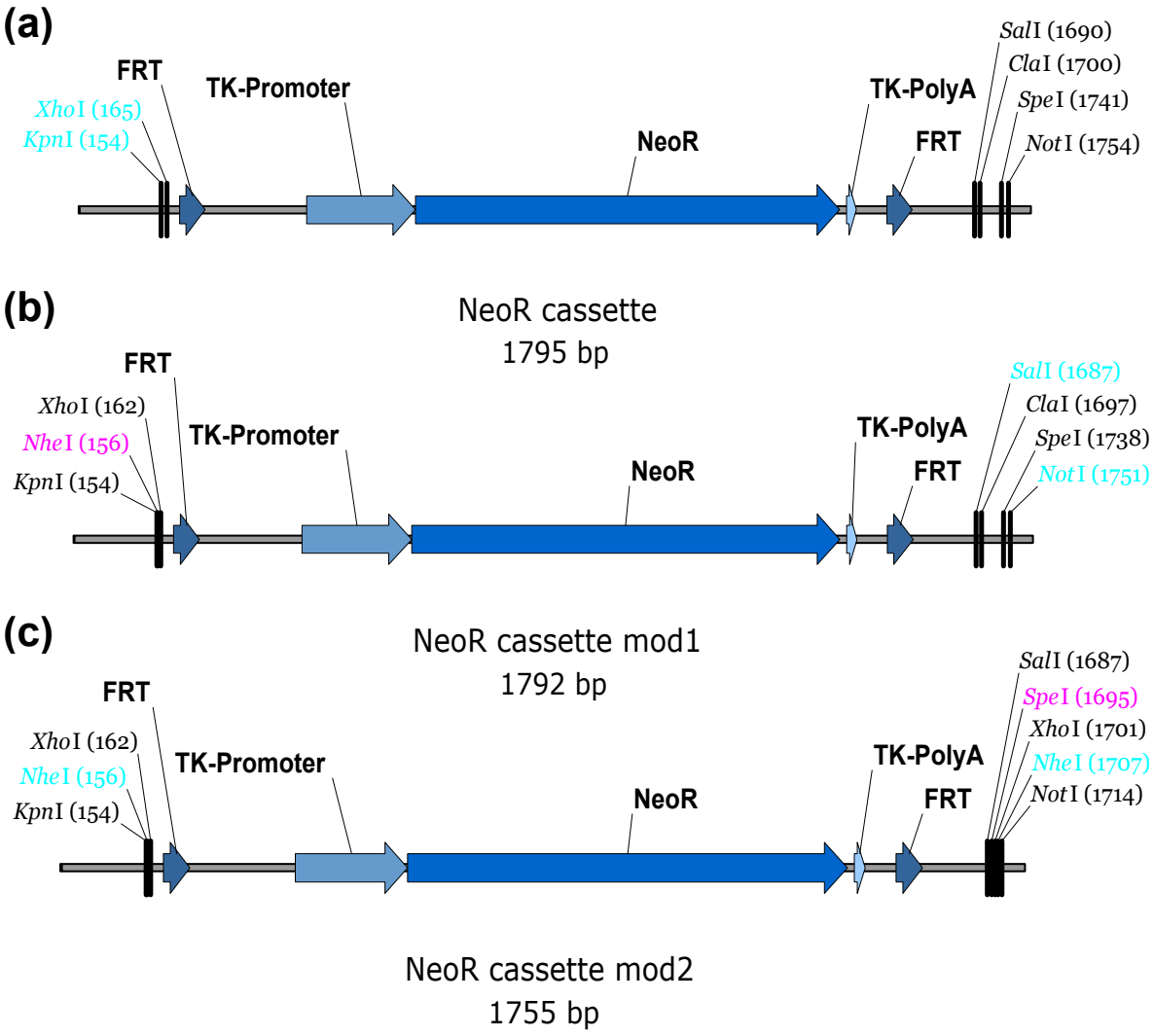


Fig. 4-8: Modification of the neomycin resistance cassette. In (a) is a part of the vector pBlu2SK-neo shown containing the *FRT* flanked neomycin resistance cassette together with adjacent restriction sites of pBluescript II SK+ important for the modification. (b) and (c) show the resistance cassette after the first and the last modification step, respectively. Sites marked in blue are indicative for the next cutting step, while sites marked in pink are newly introduced sites, which are important for the cloning of the targeting vector.

4.3.6. Cloning of the targeting vector

For the final cloning of the targeting vector a fragment of genomic DNA was needed that contained the entire Rag1 locus. Therefore a database search with the Rag1 mRNA sequence (GenBank #M29475) against the NCBI HTG (high throughput genomic) sequences at the RZDP Deutsches Ressourcenzentrum für Genomforschung (http://www.rzpd.de) was performed. The HTG division contains 'unfinished' DNA sequences generated by the high-

throughput sequencing centers and was established to provide these 'unfinished' genomic sequence data rapidly to the scientific community. The screening recovered a clone from a genomic BAC (bacterial artificial chromosome) library (clone RP23-111E15, GenBank #AC084753), that should contain the Rag1 locus. This was confirmed by an additional search within a nucleotide database using Fasta nucleotide (http://www.ebi.ac.uk/fasta33/nucleotide; Pearson and Lipman, 1988). Subsequently, the clone was obtained from RZPD. This BAC clone was already sequenced in parts. Thus it was possible to align all known elements to assemble a map of the complete Rag locus (Fig. 4-11a). With this map a restriction fragment for further cloning could be selected. As the best choice a 9.6 kb BamHI fragment became apparent.

The knowledge of the sequence of the Rag locus made it also possible to search for potential probes for the Southern blot analysis described above. For this search, repetitive DNA regions were identified by the program CENSOR (http://www.girinst.org/Censor_Server-Data_Entry_Forms.html; Jurka *et al.*, 1996) and were excluded from the probe design. HYB-1, HYB-5B and HYB-3 were chosen this way to function as external 5'and 3' and as internal probes, respectively (Fig. 4-6; Fig. 4-7).

After receiving the BAC clone stock from the RZPD, the identity of the fragment was confirmed first. This was tested by PCR with *Rag1* specific primers on isolated BAC DNA. Both primer pairs showed a product of the expected size, thus verifying that the right genomic fragment constituted the BAC clone (Fig. 4-9).

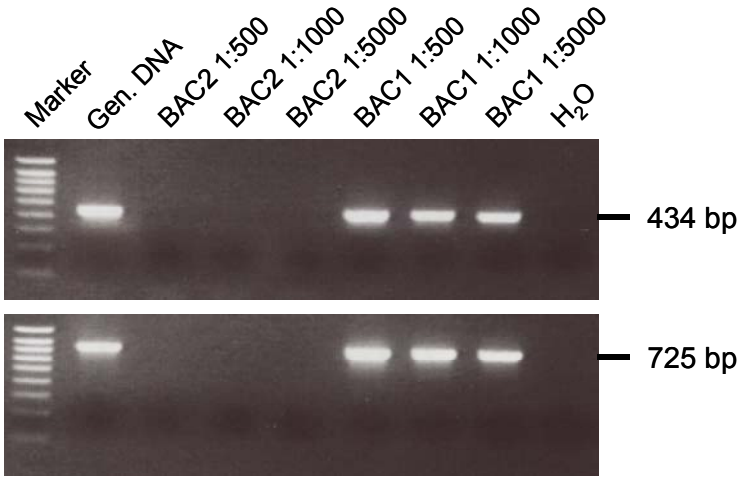


Fig. 4-9: Confirmation of the BAC clone. Isolated DNA from the ordered BAC clone (BAC1) was tested by PCR in different dilutions with two primer pairs specific for *Rag1*. As negative control served another independent BAC clone (BAC2). As positive control DNA from a tail biopsy of C57BL/6 mice was taken. The expected bands were 434 bp and 725 bp in size, respectively.

These primers were also useful for the first cloning step in the generation of the targeting vector. BACs contain large fragments of genomic DNA (40-300 kb) and it is sometimes difficult to subclone a specific restriction fragment. With the help of a colony PCR on the other hand, it was possible to identify clones which had integrated the correct fragment into their vector backbone amongst several other colonies.

(a)

SacI-NotI-BamHI-AatII-~~KpnI~~

```
5'  GAGCTC^GCGGCCGC^GGATCC^GACGTC^TGTACC  3'
3'  CTCGAG^CGCCGGCG^CCTAGG^CTGCAG^ACATGG  5'
```

(b)

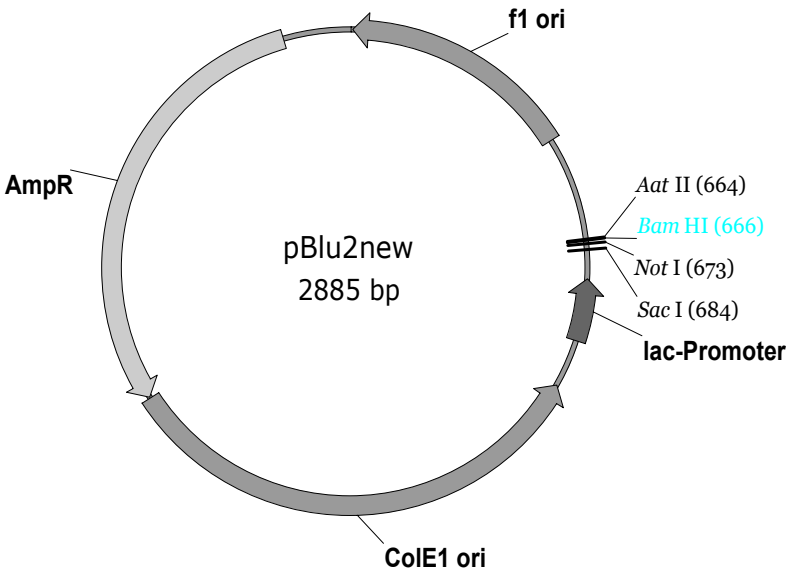


Fig. 4-10: Generation of pBlu2new. The multiple cloning site of the vector pBluescript II SK + was replaced by the annealed oligonucleotides shown in (a). Black and pink nucleotides represent the sequence of the oligonucleotides, while blue nucleotides only serve to elucidate the restriction sites. Pink nucleotides do not belong to the recognition sequence of the enzyme, which leads to the destruction of the restriction site after integration of the annealed oligonucleotides. The newly generated vector with the reduced multiple cloning site is displayed in (b). The blue BamHI site is indicative for the next cutting step.

The 9.6 kb BamHI fragment was first cloned into the vector pBluescript II SK + and the entire CDS region was sequenced. The sequencing revealed one difference to the mRNA sequence found in the database. This nucleotide variance should lead to a predicted amino acid exchange of histidine to leucine at amino acid position 609. Therefore, the same region was amplified out of genomic DNA from a C57BL/6 mouse and sequenced. An identical nucleotide difference was also found here. Thus, this difference is most likely a sequencing

error in the mRNA sequence of the database or possibly a genetic polymorphism between different mouse strains. Since the sequence of the *Rag1* gene recovered from the BAC clone was also found in the genome of C56BL/6 mice, this *Rag1* gene was considered functional and the subcloned fragment was taken for further cloning.

The 9.6 kb BamHI fragment was then integrated into a descendant of the Bluescript vector, pBlu2new (Fig. 4-10b). To generate this vector, the MCS of the original pBluescript II SK + was removed by a SacI/KpnI digestion and replaced by a new one, which was specifically designed for this particular targeting (Fig. 4-10a).

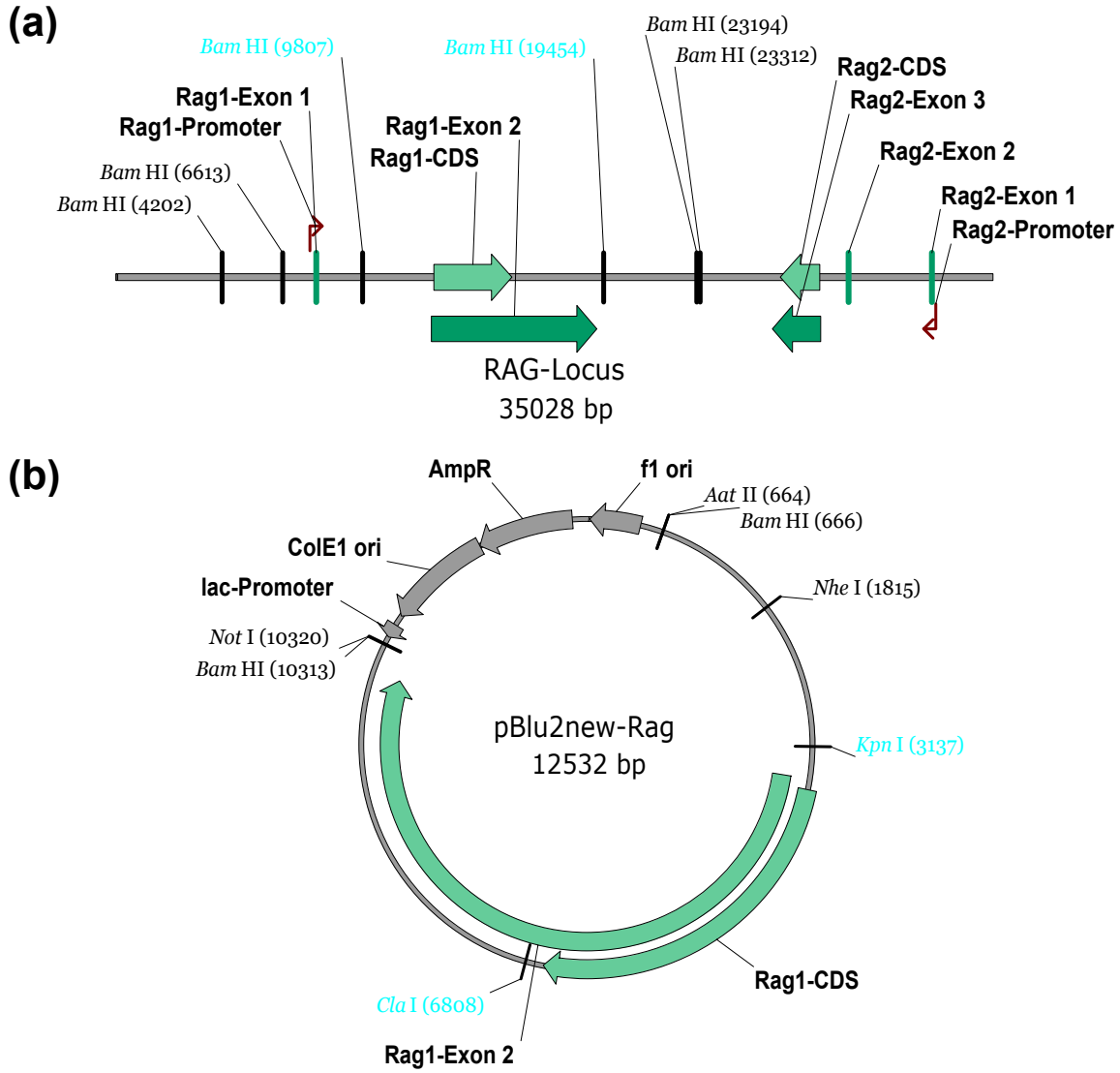


Fig. 4-11: Subcloning of the genomic fragment. The wild-type Rag locus is shown in (a). Promoters, exons and coding sequences of *Rag1* and *Rag2* are indicated. Restriction sites of BamHI are displayed, those marked in blue bound the subcloned genomic fragment. The subcloning of the BamHI fragment into pBlu2new leads to pBlu2new-Rag (b). Only some restriction sites are shown. Those marked in blue are indicative for the next cutting step.

It was important to destroy or delete some restriction sites of the old MCS, because otherwise they would interfere with further cloning steps. For instance, a nucleotide replacement in the oligonucleotides led to a destruction of the KpnI site. BamHI was introduced again for the insertion of the genomic fragment of DNA. While AatII or NotI were first thought as alternative sites for linearization of the construct, NotI was later taken for cloning of the DTA cassette. The insertion of the BamHI fragment into pBlu2new resulted in pBlu2new-Rag (Fig 4-11b). Clones that had integrated the *Rag1* exon in the same transcriptional orientation as the lac-promoter grew very poorly and the DNA yield of plasmid preparations was very low, possibly due to transcriptional effects that resulted in a metabolic burden for the transfectants. Therefore, a clone was chosen for further cloning which had inserted the *Rag1* gene in the opposite orientation.

Before it was possible to continue with the next cloning step, the plasmid DNA was first transformed into the *dam*$^-$ E. coli strain INV110, because the ClaI site, that was supposed to be used in this step, was found to be methylated by the Dam methylase and ClaI is a methylation sensitive enzyme. After recovery of pBlu2new-Rag from *dam*$^-$ bacteria, it was possible to remove the CDS region by a ClaI/KpnI digestion and replace it by an adapter leading to pBlu2new-loxinv (Fig. 4-13b).

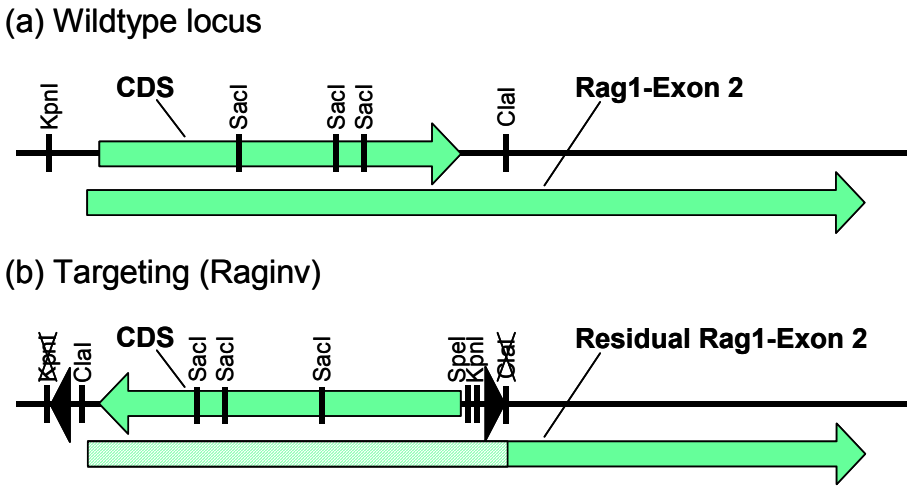


Fig. 4-12: The *loxP* flanked region. The wild-type locus containing exon 2 of the *Rag1* gene is shown in (a). The restriction sites KpnI and ClaI used for cloning are indicated. The targeted locus is shown in (b). The original restriction sites KpnI and ClaI are destroyed. At these sites the *loxP* sites were introduced in opposite orientation (indicated by arrowheads). Together with the new restriction sites an SpeI site was inserted. The CDS is now inverted preventing thus *Rag1* expression. Note that the SacI sites are distributed asymmetrically in the CDS and could therefore used together with a site outside the CDS as restriction sites for Southern blot analysis.

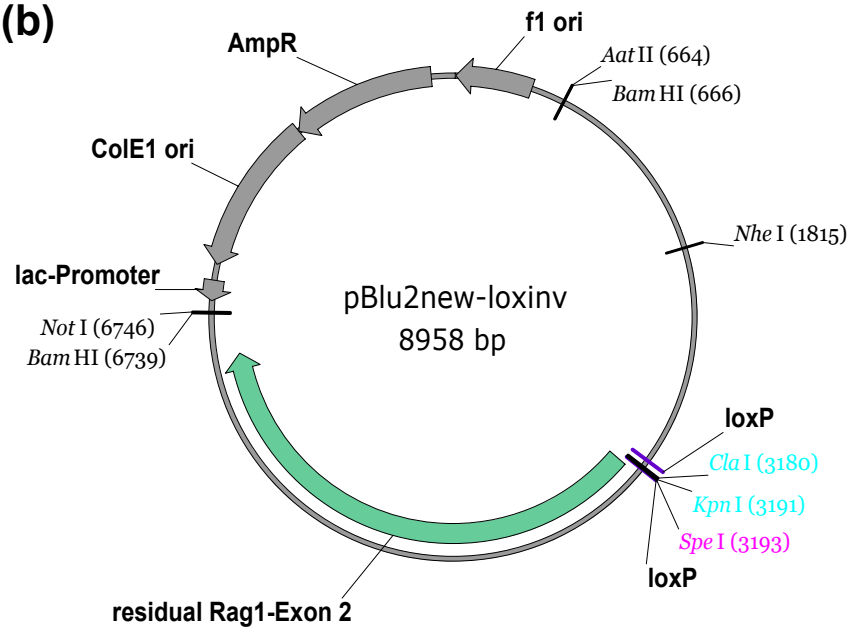
(a)

~~KpnI~~-**LoxP**-NdeI-ClaI-2 bp-KpnI-SpeI-**PxoL**-~~ClaI~~

```
5' GGTACT^ATAACTTCGTATAGCATACATTATACGAAGTTAT^CATATG^ATCGAT^AT
3' CCATGA^TATTGAAGCATATCGTATGTAATATGCTTCAATA^GTATAC^TAGCTA^TA

^GGTACC^ACTAGT^ATAACTTCGTATAATGTATGCTATACGAAGTTAT^ACCGAT 3'
^CCATGG^TGATCA^TATTGAAGCATATTACATACGATATGCTTCAATA^TGGCTA 5'
```

(b)



(c)

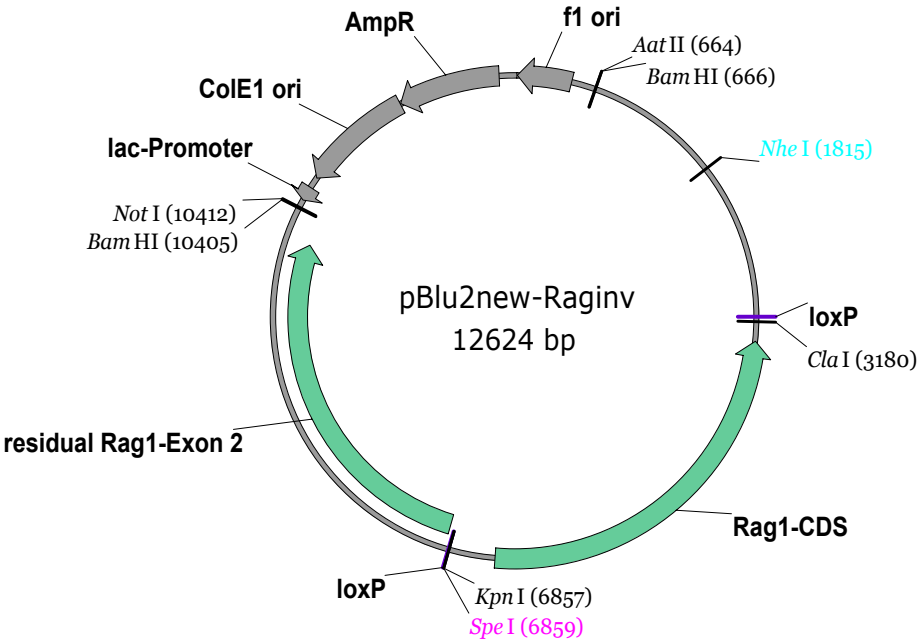


Fig. 4-13: Cloning steps leading to the inversion of the CDS. The coding sequence of the vector pBlu2new-Rag was replaced by the annealed oligonucleotides shown in (a). The orientation of the *loxP* sites becomes visible from the underlined ATG. This cloning leads to pBlu2new-loxinv (b). After this step the CDS is reintroduced, now in inverted orientation due to the new ClaI and KpnI sites, leading to pBlu2new-Raginv (c). Blue sites are indicative for the next cutting step, while the newly introduced SpeI site, important for the Southern strategy, is marked in pink.

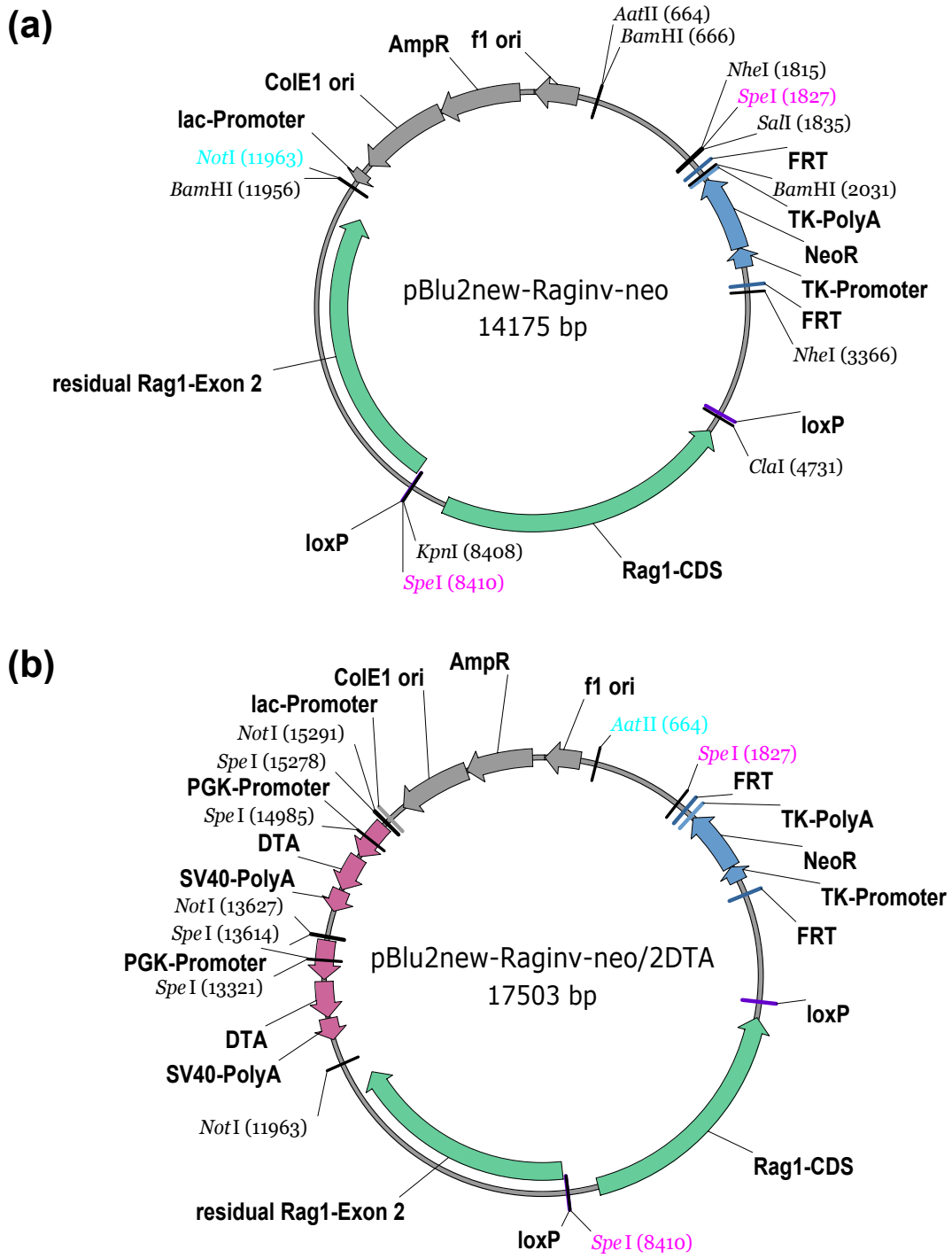


Fig. 4-14: Final introduction of positive and negative selection markers. The neomycin resistance cassette was cloned into the NheI site of the vector pBlu2new-Raginv resulting in pBlu2new-Raginv-neo (a). Two DTA cassettes were then cloned into the NotI site leading to the final targeting vector pBlu2new-Raginv-neo/2DTA (b). The AatII site for the linearization of the vector is marked in blue, while the newly introduced SpeI sites, important for the Southern strategy, are marked in pink.

This adapter was generated using annealed oligonucleotides (Fig. 4-13a). It was designed in a way that its integration destroys the ClaI and KpnI sites and adds new ClaI and KpnI sites sites further inside (Fig. 4-13a). In addition, it contained two *loxP* sites in opposite orientation which becomes obvious from the orientation of the ATGs in the spacer region (Fig. 4-13a). It was important to insert these ATGs in an orientation which does not lead to translation of the mRNA that will be produced from the modified gene. Furthermore, an SpeI site was introduced with the adapter into the targeting vector important for the later screening by Southern blot analysis (Fig. 4-13a).

The Dam methylase methylated again the ClaI site, that should be used in the next cloning step, in the new segment. Therefore, pBlu2new-loxinv was transformed into the INV110 strain to obtain unmethylated DNA for the next cloning step.

For this step the ClaI/KpnI fragment, containing the *Rag1* CDS excised before, was cloned into the vector pBlu2new-loxinv resulting in pBlu2new-Raginv (Fig. 4-13c). The order of the sites in the adapter resulted in an inverted orientation of the CDS in the targeting vector and this inverted CDS was now flanked by *loxP* sites in opposite orientation (Fig. 4-12).

The neomycin resistance cassette was then excised from pBlu2SK-neo with NheI and inserted into the NheI site of the pBlu2new-Raginv resulting in pBlu2new-Raginv-neo (Fig. 4-14a). A clone was chosen which had the transcriptional orientation of the neomycin resistance cassette opposing the normal *Rag1* transcription. At this step, the whole sequence of the neomycin resistance cassette and the CDS together with the flanking *loxP* sites was controlled by sequencing.

Initially it was not planed to use a negative selection marker, because some groups called the profit of this step in question. However, this decision was later revised and shortly before the transfection of the ES cells the DTA cassette of pGEM-DTA (Fig. 2-3) was cloned into the NotI site of the targeting vector, originally planed for linearization. A clone was inadvertently chosen for DNA preparation which had integrated two DTA cassettes in the same orientation (Fig. 4-14b). For time pressure, it was not possible to expand another clone. In addition, there was no argument against the use of a second DTA cassette. Thus, this vector was taken as targeting vector for the ES cell transfection. The high frequency of homologous recombinants, obtained in the transfections, might actually argue in favor of the use of two DTA cassettes.

4.3.7. Functional test of the *loxP* sites in *E. coli*

Since the procedures required to establish a mouse strain from recombinant ES cells are rather time consuming, the functionality of some components of the construct were tested in alternative systems. The recombination competence of the *loxP* sites in the targeting construct was tested in a Cre expressing *E. coli* strain. Hereby, the vector pBlu2new-Raginv-neo/2DTA was transformed into the strain 294-Cre (Buchholz *et al.*, 1996a), which had the *Cre* gene integrated into its genome. The expression of the recombinase was under the control of a temperature-sensitive repressor, leading to a functional enzyme when incubated at 37°C.

The plasmid DNA from particular clones, after the transformation, was isolated and digested with SpeI (Fig. 4-15). The targeting vector, designated as construct in Fig. 4-15 and used as control, showed the expected fragments for the non-recombined vector. The next two lanes display two independent clones, which showed two additional bands of 2909 and 8585 bp, indicative for a recombination. The bands of 4911 and 6583 bp were still present, since the recombination of *loxP* sites in opposite orientation is a continuous process, whereby the intervening segment is flipping with respect to the rest of the molecule in the presence of a functional Cre recombinase. The restriction pattern of clone 3 could be explained by a deletion of the intervening segment instead of an inversion. It is known that high expression levels of Cre, as found in 294-Cre at 37°C, might alter the directionality in Cre-*loxP* site-specific recombination and result in deletions instead of inversions (Aranda *et al.*, 2001).

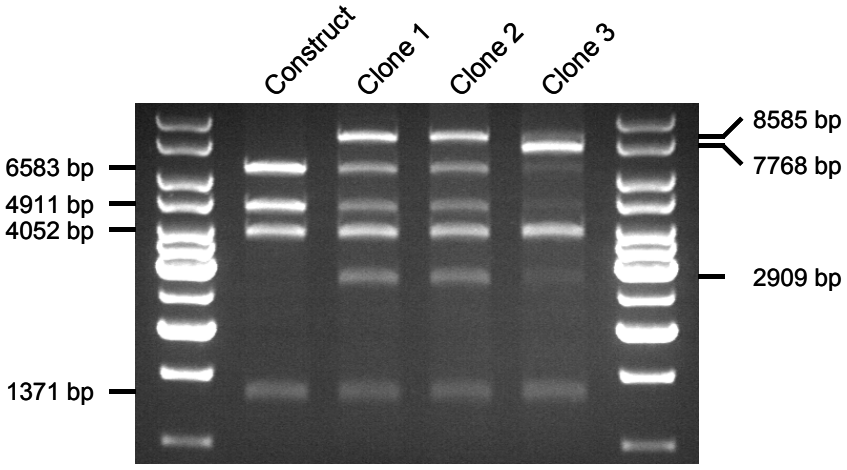


Fig. 4-15: Site-specific DNA recombination in *E. coli*. The targeting vector was transformed into the *E. coli* strain 294-Cre. Plasmid-DNA was isolated from three clones (Clone 1, 2 and 3) and digested with SpeI. The targeting vector was digested as control (Construct). The sizes of the bands are indicated besides the photo. All digestions showed an expected band of 293 bp, not represented on this photo.

4.3.8. Homologous recombination in ES cells

The planned background for the Rag1 knock-in mouse was initially BALB/c, because a mouse model, the L2 mouse (Engel *et al.*, 1998), intensively studied in the group with respect to B1 cell development and function, was also on the BALB/c background. However, since the genomic DNA used for the targeting vector was derived from C57BL/6 mice, and since it was not clear whether potential polymorphisms between the different mouse strains could influence the chance of recombination, the targeting was performed both, in BALB/c and in C57BL/6 derived ES cell lines. Another argument for the use of C57BL/6 derived ES cells was that most of the Cre transgenic mouse strains, that should be used later for experiments with the newly established mouse strain, are mainly established on the C57BL/6 background. Thus a homogenous genetic background could be maintained when crossing such mouse strains.

After transfection of BALB/c (BALB/c background) and Bruce-4 (C57BL/6 background) ES cells and subsequent selection with G418, 60 clones for BALB/c and 24 clones for Bruce-4 were picked and frozen at the 96 well plate stage. Southern blot analysis using the 5' probe HYB-1 (data not shown) revealed 4 and 2 homologous recombinants, respectively (Table 4-3). Southern blot analysis after expansion of the clones, using the 3' probe HYB-5B indicated that some of the homologous recombinants were only partial recombinants. They had only integrated the neomycin resistance cassette but not the second SpeI site at the second *loxP* site. Only a band of 10.5 kb was seen, in addition to the 14.1 kb wild-type band, for the C57BL/6 clone 6-2 (Fig. 4-16). Similar results were obtained from the three BALB/c derived partial recombinants (data not shown). However, the C57BL/6 clone 22-2 and the BALB/c clone 23 exhibited the expected 3.9 kb band for a complete integration of the floxed inverted CDS (Fig. 4-16 and Fig. 4-17). Therefore these two clones were homologous recombinants.

Table 4-3: Efficiency of homologous recombination.

ES cell line	Genetic background	No. of picked clones	Homologous recombinants	Complete homologous recombinants
BALC/c	BALB/c	60	4	**1**
Bruce-4	C57BL/6	24	2	**1**

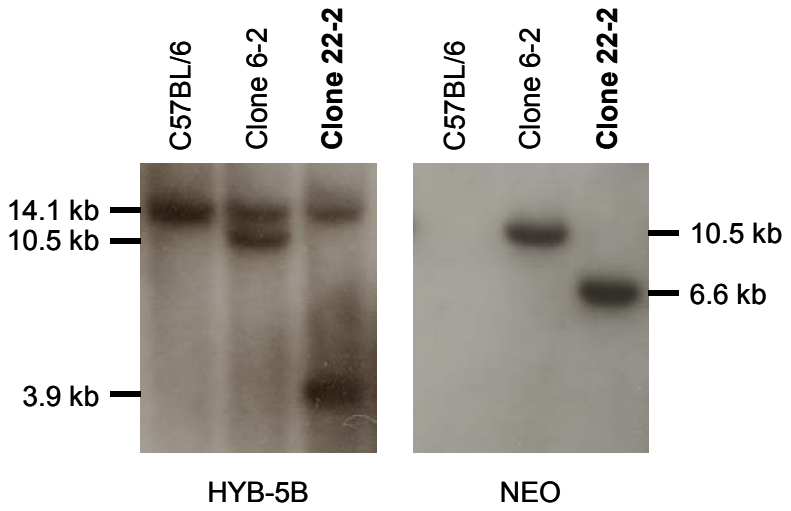


Fig. 4-16: Southern blot analysis of C57BL/6 ES cell clones. Genomic DNA from wild-type Bruce-4 cells (C57BL/6) and clones 6-2 and 22-2 were digested with SpeI and hybridized with probe HYB-5B to verify the targeting event and NEO to exclude additional transgene integration, respectively.

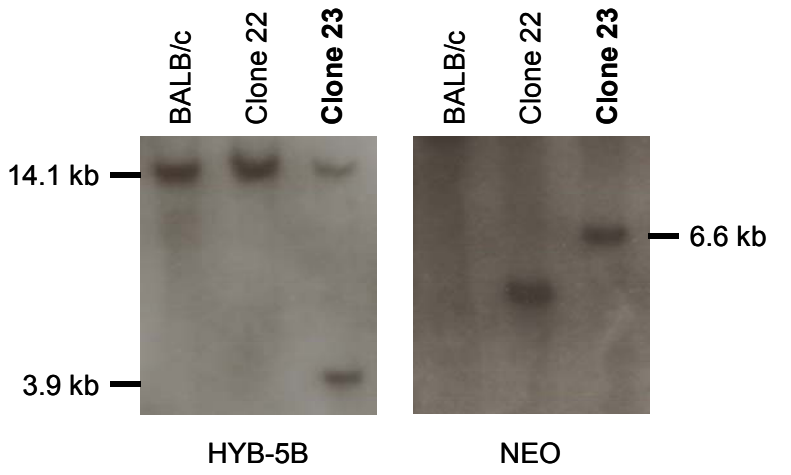


Fig. 4-17: Southern blot analysis of BALB/c ES cell clones. Genomic DNA from wild-type BALB/c cells (BALB/c) and clones 22 and 23 were digested with SpeI and hybridized with probe HYB-5B to verify the targeting event and NEO to exclude additional transgene integration, respectively.

The frequencies of these recombinants were 1 out of 24 and 1 out of 60, respectively. Such high frequency was not expected from the initial targeting experiments of the Rag1 locus (Mombaerts *et al.*, 1992), and might be due to the presence of the second DTA cassette.

To exclude the possibility that the clones had integrated an additional copy of the construct as transgene, additional hybridization with a probe specific for the neomycin resistance cassette (NEO) was performed. Both clones showed only a single band of the expected 6.6 kb when hybridized with the neo probe (Fig. 4-16 and Fig. 4-17). Thus both could be used for establishing the new mouse model.

4.3.9. Functional test of the *loxP* sites *in vitro*

The recombination competence of the *loxP* sites in the context of a mammalian cell was tested in an *in vitro* assay using the C57BL/6 derived clone 22-2. In this assay a His-TAT-NLS-Cre protein (Peitz *et al.*, 2002) was used to transduce the cells directly. This Cre protein was histidine-tagged and fused to a nuclear localization signal (NLS) in addition to a peptide derived from the HIV TAT protein. This combination results in enhanced cellular uptake, transport to the nucleus and subsequent site-specific recombination. Different fusion protein concentrations were tested to find out an optimal amount without toxic effects that might emerge with high Cre concentrations for the cells. One of these concentrations gave rise to recombination events *in vitro*. The expected band of 2.9 kb was present at a Cre concentration of 0.25 µM indicating functionality of the *loxP* sites also in the mammalian system (Fig. 4-18).

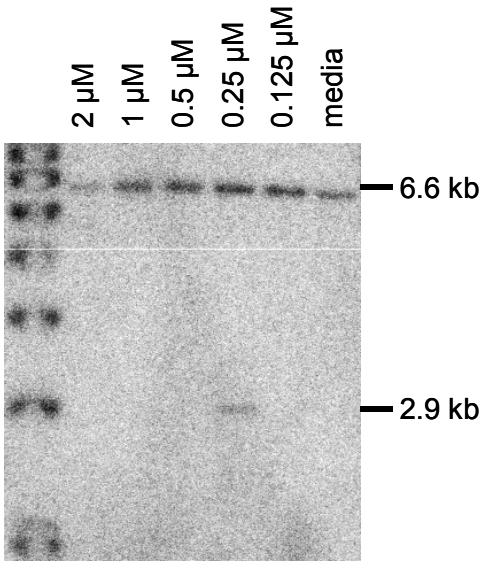


Fig. 4-18: Site-specific DNA recombination after Cre-transduction *in vitro*. The C57BL/6 ES cell clone 22-2 was transduced with different concentrations of Cre protein or media as control *in vitro*. After expansion of the transduced cells, genomic DNA was isolated, digested with SpeI and hybridized with probe NEO to verify the recombination event.

4.3.10. Deletion of the neomycin resistance cassette in ES cells

The neomycin resistance cassette could later interfere with gene function, either directly or due to its constitutive promoter; therefore it needed to be removed. This could be carried out still in the ES cells *in vitro* or later in the mice *in vivo*. For the *in vitro* deletion in ES cells a second transfection is necessary, but each transfection minimizes the chance to obtain germline transmission. Since a Flp-e transgenic mouse already exists (Rodriguez *et al.*, 2000), which is backcrossed on the C57BL/6 background for several generations, it was decided that the cassette should be removed *in vivo* for the C57BL/6 derived clone 22-2. Since such a transgenic mouse does not exist with BALB/c background, the neomycin resistance cassette

from the BALB/c derived ES cell clone was removed *in vitro*. Although not necessary *in vitro*, this removal was also performed for the C57BL/6 derived ES cell clone 22-2.

Both clones were transfected with a Flp-e expression plasmid (kind gift of Thomas Wunderlich, University of Cologne). This plasmid expresses the mutated form of the Flp recombinase that is active at 37°C. After transfection and growth for a few days, clones were picked and tested as triplicates for G418 sensitivity. G418 sensitive clones were expanded and tested by Southern blot analysis. Both C57BL/6 and BALB/c derived ES cells gave rise to three clones which had deleted the neomycin resistance cassette (Table 4-4). They all showed the expected 5.2 kb recombinant band in addition to the 14.1 kb wild-type band (Fig. 4-19). The BALB/c derived clone 1-F7 showed also the non-deleted 6.6 kb band indicative for a contaminated clone. This was also apparent in the test for G418 sensitivity, since resistant cells survived the selection.

Table 4-4: Efficiency of *in vitro* deletion of the neomycin resistance cassette.

ES cell clone	Genetic background	No. of picked clones	G418 sensitive clones
23	BALB/c	192	3
22-2	C57BL/6	384	3

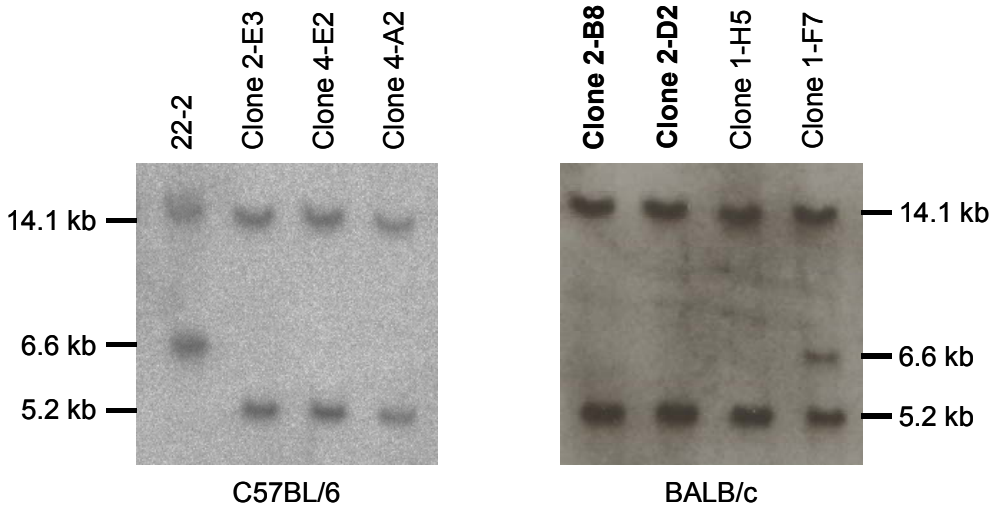


Fig. 4-19: *In vitro* deletion of the neomycin resistance cassette. The C57BL/6 ES cell clone 22-2 and the BALB/c ES cell clone 23 were transfected with a Flp-e expression plasmid. Genomic DNA from G418 sensitive clones were digested with SpeI and hybridized with probe HYB-3 to verify the deletion of the neomycin resistance cassette.

4.3.11. Chimeric mice and their offspring

The clone 22-2 (C57BL/6 background) and the clones 2-D2 and 2-B8 (BALB/c background, neomycin resistance cassette deleted) were chosen for injection into blastocysts. These blastocysts or donor embryos were derived for clone 22-2 from CB20 mice while those for 2-D2 and 2-B8 were derived from C57BL/6 mice. This combination allows to distinguish later between donor derived and ES cell derived offspring based on the coat-color (see below). Injected ES cells could be pluripotent and would give rise to all tissues like skin, muscles and organs. If they have retained their omnipotency they could differentiate into germ cells, in addition, and the new allele would be hereditary. Because ES cell lines are usually derived from male mice, the injection of ES cells should mainly result in male offspring when they contribute to the germline.

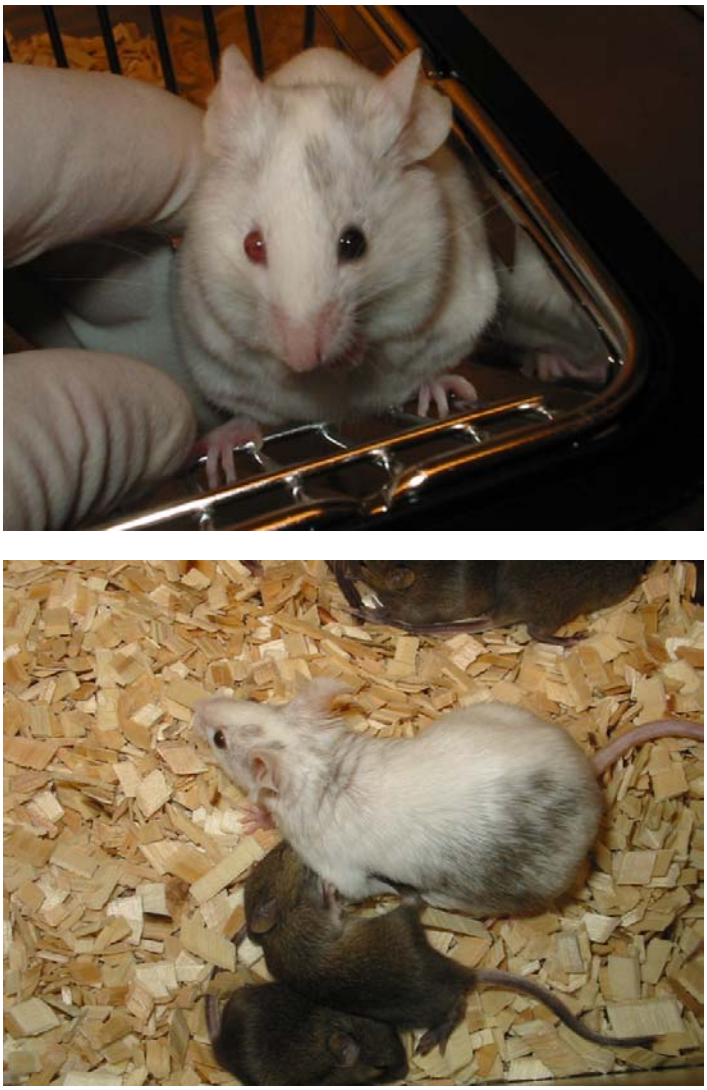


Fig. 4-20: Chimera derived from the C57BL/6 ES cell clone 22-2. Shown is a female chimera with 50% chimerism (applied to the coat-color) together with its agouti offspring.

The blastocysts were transferred after injection into foster mice. Some of their offspring showed coat-color chimerism indicating successful transfer and differentiation of the ES cells (Table 4-5 and Fig. 4-20).

Table 4-5: Overview of the different blastocyst injections.

No.	Clone	Background	Blastocysts	Pups	Chimeras		Range[a]
					M	F	
1	22-2	C57BL/6	53	7	3	1	50-90%
2	2-D2	BALB/c	76	18	4	2	25-70%
3	2-B8	BALB/c	26	2	-	-	-
4	22-2	C57BL/6	44	22	10	5	10-80%

[a] Range of chimerism applied to the coat-color.

These chimeric mice were then bred to wild-type mice i.e. C57BL/6 for 22-2 and BALB/c for 2-D2. Dependent on the coat-color of the offspring it was possible to decide if germline transmission was obtained from the chimera or not. Thus, the combination for the clone 22-2 with C57BL/6 mice will give rise to black animals, when the offspring is derived from the ES cells, while offspring between C57BL/6 and the CB20 donor will result in brown-gray animals due to the dominant agouti gene in the donor mice. Analogue for the clone 2-D2, the agouti gene of the BALB/c mice in combination with the donor derived C57BL/6 germ cells should lead to an agouti offspring, while white offspring will be derived from the ES cell derived sperm cells.

Only a small fraction of the chimeras gave rise to a black or white offspring, respectively. Nevertheless, this indicated that germline transmission was obtained for both ES cell clones (Table 4-6).

Table 4-6: Overview of the chimeras that showed coat-color germline transmission.

No.	Clone (Background)	Chimerism	Litters	Pups	Germline transmission	
					Coat-color	Targeted allele
1	22-2 (C57BL/6)	80%	1	4	4	3
2	22-2 (C57BL/6)	90%	6	53	2	-
3	2-D2 (BALB/c)	50%	3	5(7)[a]	5	1

[a] Two pups died short after birth.

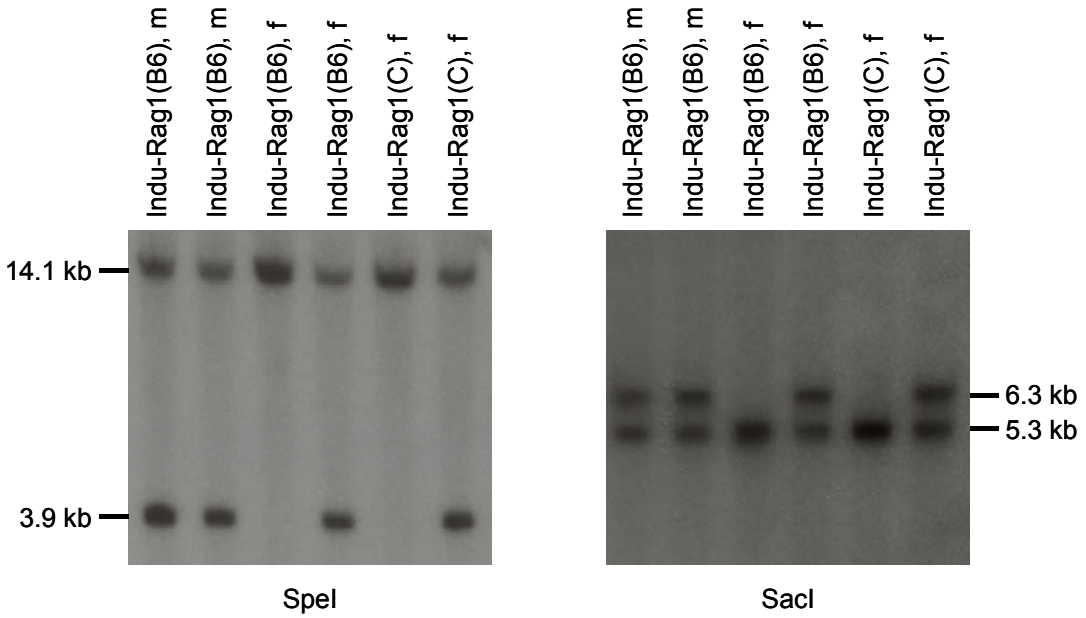


Fig. 4-21: Southern blot analysis of the offspring that showed coat-color germline transmission. Genomic DNA from the offspring of both strains were digested with SpeI and SacI, respectively, and hybridized with probe HYB-5B for the detection of the targeted allele.

To find out whether the targeted chromosome was transmitted to the ES cell derived offspring, Southern blot analysis was employed. Due to the heterozygocity of the targeted ES cells, only half of the offspring is expected to carry the particular allele. DNA from tail biopsies was digested with SpeI and SacI respectively and hybridized with the 3' probe HYB-5B. For both clones, mice were found, that carried the targeted allele. These mice showed the expected 3.9 kb SpeI restriction fragment and the 6.3 kb SacI restriction fragment in addition to the wild-type band (Fig. 4-21).

These mice were taken for breeding to establish two new mouse strains. The newly established mouse strains were named Indu-Rag1 from inducible Rag1 combined with the ending (B6) or (C) indicative for the genetic background (B6 for C57BL/6 and C for BALB/c). The heterozygous Indu-Rag1 mice were fertile and were able to transmit the targeted allele. Crossing with additional recombinant mice, with inducible Cre expression, this newly generated system will now allow definitive experiments to study differentiation, antibody repertoire establishment and selection processes during B cell development in the adult bone marrow.

5. Discussion

In this work different aspects of B cell ontogeny were investigated. First, differential expression of germline transcripts from both light chain loci was measured as diagnostic marker for the regulation of accessibility of the particular loci for rearrangement. Second, gene expression patterns of B cell precursors from either fetal liver or adult bone marrow were compared using microarrays, thus significantly extending previous data. Third, a new mouse model with inducible lymphopoiesis was established, that should provide further insights into the different ontogeny between fetal liver and bone marrow derived B cell precursors.

5.1. Germline transcripts of immunoglobulin light chain variable regions are structurally diverse and differentially expressed

By using 3'-RACE technology it was possible to isolate germline transcripts of 17 V_κ gene segments that could be assigned to 12 of the 18 V_κ families. Transcripts from V regions that were oriented in either transcriptional direction could be recovered during these experiments (Table 4-1). Additionally, germline transcripts of all three V_λ gene segments were isolated this way.

Only germline transcripts of functional or potentially functional V_κ gene segments were obtained. None of the isolated transcripts could be assigned to a pseudo-gene. This is probably partially due to the way the primers were designed, since the primers in most cases covered sequences of functional or potentially functional gene segments. In addition, the sample size of the germline transcripts obtained so far is still very small. Thus, together with the primer selection this might explain why no amplification products of pseudo-gene transcripts were obtained. On the other hand, it can not be ruled out that transcripts of pseudo-genes are absent or present at extremely low levels, since many of these gene segments display alterations in their promoter regions (Thiebe *et al.*, 1999; Schable *et al.*, 1999). In addition, the half-life of some of these RNAs could be extremely short which might also be the case for some of the functional V_κ segment transcripts. Finally, some V gene segments might lack polyadenylation sites. Such transcripts would escape detection because cDNA synthesis was started from the polyA tail. Since the 3' regions of only some of the V_κ gene segments are published, this possibility can not yet be excluded. Together, this might explain

why, for members of certain V_κ families, no amplification products were obtained despite of extensive trials. As germline transcripts of Ig genes are believed to have no function, the transcripts we observed might just be the result of the opening of particular gene segments. Hence, instability and low abundance of germline transcripts would have no physiological consequences.

The extreme heterogeneity in size of germline V transcripts is striking. The lengths of the 3'-UTRs were found to be between 41 and 670 nucleotides. In addition, a few transcripts were polyadenylated at two or even three alternative sites. 3'-UTRs within families, where a comparison was possible, were either extremely homologous in sequence and size (V_κ01) or very different in both aspects (V_κ08 and V_κ19/38). The observed extreme heterogeneity only adds to the notion, that germline transcripts might have no function, and the lack of selective constraints would allow for variability of these 3'-UTRs. On the other hand, conserved 3'-UTRs were found in some families (V_κ01 and V_λ1/2). Such sequence conservation might be explained by gene conversion which is known to act on multi-gene families. This recombination mechanism introduces extensive changes at sites of variability but also conserves sequences at other sites.

One might also expect to find similar size and sequence heterogeneity for the intron between the first and the second exon of a V gene segment, as such sequences might also be devoid of a function. However this seems not to be the case (Kirschbaum *et al.*, 1998; Kirschbaum *et al.*, 1999; Roschenthaler *et al.*, 1999; Roschenthaler *et al.*, 2000; Schable *et al.*, 1999; Thiebe *et al.*, 1999). Although size and sequence heterogeneity can be found when different families are compared, comparison within the same family showed that length and sequence remained highly conserved, with only a few exceptions. Since the intron is also part of the primary transcript of a functional immunoglobulin light chain after rearrangement, obviously selective constrains act on this part of the transcription unit other than the 3'-UTR of germline transcripts. In addition, so far unknown functions might be encoded in this stretch of DNA.

The previous finding that JC_κ is activated and rearranged before the λ locus was confirmed and extended (Engel *et al.*, 1999). Already at the earliest developmental stage of R2-bfl differentiation which could be analyzed, germline transcripts of JC_κ and V_κ21 were detectable. Although, the member of the most distal V_κ family V_κ02 lagged behind in its activation, it already is detectable at the same time as V_λ transcripts. Since rearrangements at the κ locus do not prevent further rearrangements at the same allele unless the most downstream J_κ5 is involved, a non-random sequential activation of V_κ gene segments from

proximal V_κ to distal V_κ regions might increase the probability that a functional heavy/light chain combination can be expressed by a B cell. This is consistent with previous findings of multiple rearrangements of the same allele at the κ locus (Yamagami *et al.*, 1999).

Besides this slight asynchronicity within the V_κ cluster and a similar asynchronicity between the V_λ and the JC_λ segments, the previous finding of a sequential activation of the κ and the λ JC cluster was now extended to the complete loci of both light chains. The slight difference in expression might be explained by the different promoters and additional regulatory elements driving the V_λ and JC_λ segments. How a differential activation within the V_κ cluster can be regulated remains to be determined. Some of the motifs of the V_κ promoters are highly conserved, while others are diverse and could influence the kinetics of activation (Bemark *et al.*, 1998). In addition, recently a transcriptional coactivator has been described to influence V_κ transcription and usage (Casellas *et al.*, 2002). However, additional regulatory elements have been determined within the λ cluster (Engel *et al.*, 2001) and so far uncharacterized DNase sensitive sites exist within the λ locus, which might indicate the existence of further regulatory elements (Hagman *et al.*, 1990). Similarly, unknown regulatory sites might also exist within the V_κ cluster.

To interpret the findings of consecutive activation of the two light chain loci and to explain the phenotype of mice in which the κ locus had been inactivated (Chen *et al.*, 1993a; Zou *et al.*, 1993), a "hit and run" hypothesis for light chain rearrangement in the mouse was proposed (Engel *et al.*, 1999). This hypothesis would also explain the κ/λ ratio found in this species (Hood *et al.*, 1967). Developing B cells at the stage of light chain rearrangement (pre-B-II; large and small) would have a limited life span. During this time they first activate their κ locus for rearrangement. Independent of the events at the κ locus, the λ locus will be activated at a consecutive developmental stage. Rearrangement of λ will take place unless the pre-B cell was already rescued to the immature B cell stage by a functional B cell receptor, resulting from a functional and appropriate κ rearrangement. This would also result in a down-regulation of the rearranging mechanism. Due to the limited life span of such pre-B cells, unsuccessful κ rearrangement can only partially be compensated by rearrangement at the λ locus, thus partially explaining the κ/λ ratio of the mouse. This predicts that extension of the life span of a pre-B cell would alter the κ/λ ratio. Mice transgenic for the anti-apoptotic gene bcl-2 indeed have an altered κ/λ ratio with a predicted increase in the frequency of λ bearing cells (Lang *et al.*, 1997). Variation in the life span of pre-B-II cells could thus explain

the various κ/λ ratios found in species other than the mouse. The synchronous activation of the V and JC segments of a particular locus described here, represent an essential prerequisite of this hypothesis.

5.2. Comparison of gene expression patterns between B cell precursors derived from fetal liver and adult bone marrow

In addition to the function of germline transcripts, many other aspects of B cell development, like the different ontogeny in fetal liver and adult bone marrow, are largely unknown. The present work describes gene expression patterns of early B cell precursors ($CD19^+$c-kit^+) derived from fetal liver and adult bone marrow. Such precursors obviously give rise to distinct B cell populations (Herzenberg, 2000). With this study it was possible to obtain significant information about differentially expressed molecules in such precursors.

Relatively few genes were found differentially expressed, but the validity of this gene expression data set was underscored by the finding that genes whose expression patterns have been identified earlier, were detected in a manner concordant with the previous results. Both TdT (Li *et al.*, 1993) and the myosin light chain (Oltz *et al.*, 1992) were found differentially expressed i.e. in bone marrow and not in fetal liver. In addition, MHC class II molecules were expressed solely in the bone marrow derived precursors (Lam and Stall, 1994; Hayakawa *et al.*, 1994). It has been suggested, that such distinct programs of class II expression during fetal and adult lymphopoiesis could result in differences in susceptibility to tolerance (Hayakawa *et al.*, 1994).

The initial analysis was based on differentially expressed genes that differ by a factor of two or more. Although a regulation factor of two seems rather low, several examples have demonstrated a functional significance of mono- versus bi-allelic expression i.e. of a twofold difference in expression levels (Nutt *et al.*, 1999b).

Other genes, however, might require more significant changes in expression level to become functionally relevant. Therefore, not all of the identified genes may actually contribute to the fate of the B cell precursors. As an example, the importance of a particular expression level for B cell development was shown for the transcription factor PU.1. High levels of PU.1 result in the differentiation of precursors into myeloid lineage cells, lower levels favor generation of B lineage cells (DeKoter and Singh, 2000). IL-7Rα was identified as critical B

cell target of PU.1. At the molecular level, low PU.1 concentrations activate the IL7-Rα gene in contrast to high PU.1 levels, which prevent IL7-Rα expression (DeKoter *et al.*, 2002).

One differentially expressed gene, found in the analysis of the array data, which appeared of particular significance, was Notch1, which showed higher expression levels in fetal liver compared to bone marrow derived precursors. Notch1 plays an important role in the T versus B lineage decision, where it is essential for the development of both thymus-dependent and -independent T cells (Radtke *et al.*, 1999; Wilson *et al.*, 2000). Bone marrow progenitors from Notch1$^{-/-}$ mice enter the thymus, but develop into B cells (Wilson *et al.*, 2001). In contrast, the expression of a dominant active form of Notch1 in bone marrow precursors leads to ectopic T cell development in the bone marrow and simultaneously blocks B cell development (Pui *et al.*, 1999). Taken together, these results suggest that Notch1 signaling must be either absent or negatively regulated in BM progenitor cells to allow B cell development, and that a Notch1 signal is sufficient for T cell commitment. It has been demonstrated that the transcription factor Pax5 essential for B lineage commitment represses Notch1 expression at the transcriptional level in B cell progenitors, providing a possible mechanism to ensure B cell development in the BM (Souabni *et al.*, 2002). Nevertheless, Notch receptors and ligands are expressed on BM progenitors and stroma as well as on FL progenitors and fetal liver. Thus, this differential expression could be of significance, especially since a molecule that might compete with Notch1, the amyloid beta (A4) precursor protein, is up-regulated in the BM. In addition, PU.1 has been identified as a specific direct target gene of Notch1, a transcription factor also involved in B cell development (Schroeder *et al.*, 2003).

Interestingly, an important role for another member of the Notch receptor family, Notch2, has been shown for the development of the MZ B cell subset. Conditional inactivation of Notch2 in the hematopoietic compartment leads to a loss of the MZ B cells (Saito *et al.*, 2003). Peritoneal B1 cells develop normally in these mice. This is in contrast to a recent report that a significant reduction in both MZ B cells and B1 cells was observed in Notch2 heterozygous mice (Witt *et al.*, 2003). The reason for this discrepancy is currently unknown.

Taken together, Notch1 appears to be a prime candidate for further detailed studies since it is a molecule that is involved in lineage decision and might thus also influence B1 versus B2 decision.

Surprisingly, in addition to the MHC class II molecules, also MHC class I molecules were found differentially expressed with low expression levels in the fetal liver derived precursors. It is known that tolerance against the fetus is a multi-factorial process. The trophoblast, which

is the outer layer of the placenta and the interface between fetal and maternal tissues, does not express classical MHC class I and class II molecules, thus making it resistant to recognition and attack of maternal T cells (Bulla *et al.*, 2003). However, low MHC class I expression levels have not been described for fetal liver derived B cell precursors before. Although the significance of this finding is still unclear, it may have relevance for practical experiments. Tissues lacking MHC class I expression are attacked by NK cells (Karre, 2002). The trophoblast might be protected from such attack by the expression of a non-classical MHC class I molecule, while in reconstitution experiments with murine cells the fetal liver derived B cell precursors could be attacked by NK cells when transferred into recipients. This hypothesis can be tested experimentally.

Unexpectedly, κ and λ light chains and an Ig gamma-2b chain were found expressed in the B cell precursors derived from the adult bone marrow. κ and λ lights chains are normally detectable at the pre-B-II stage, while Ig gamma-2b chains should only be detectable after antigen contact and class switch recombination. Detection of germline transcripts from the κ and the λ light chain loci was unlikely as explanation, because such transcripts become detectable in cells of developmental stages that were not included in this analysis and occur only at low abundance. This surprising finding might be explained by contaminating plasma cells in the sorted precursors. Plasma cells can be found in the bone marrow and they are absent in the fetal liver. Because of the high expression of genes encoding antibodies in such cells, already low numbers of contaminating cells could give rise to high mRNA levels. This possible contamination should not obscure the other data since only Ig mRNA is exceptional high in plasma cells.

In summary, the combination of the previous knowledge about B cell development and the functional annotation of differentially expressed genes defined by the microarray experiments identified genes potentially involved in specific processes that take place in the particular murine B cell precursors. Nevertheless, it is still open, whether these differences are significant or not. Sometimes only one gene of a particular pathway or a signaling cascade was found differentially expressed. Thus, obviously further detailed investigation is necessary. However, these array date provide clues, which pathways are prime candidates. As a first confirmation quantitative measurements at the RNA and protein level are required. These analyses should also include the other molecules involved in the pathways indicated.

Another obvious task will be to test the expression of the identified cell surface markers, thus possibly providing new markers for the isolation of such cells. Furthermore, functional

analysis should be performed. The availability of ApoE$^{-/-}$ and conditional VCAM-1$^{-/-}$ mice provide a first starting point. The conditional VCAM-1$^{-/-}$ mice could be crossed to CD19-Cre mice which results in deletion of VCAM-1 in B cells. Because of the differential expression of both ApoE and VCAM-1 a functional difference in the knock-out mice compared to the wild-type should become detectable.

The comparison of gene expression patterns was initially performed to provide further evidence for or against distinct B cell progenitors. However, after the present analysis the question remains whether such pro- and pre-B-I cells are the precursors in which the different B cell traits are distinct. This cell type is the first to be committed and the microenvironment appears to induce the particular trait at this stage. However, the commitment might only partially be manifested in the differentially expressed genes at the pro-/pre-B-I stage and it might become more obvious at consecutive stages of B cell ontogeny. Consequently, the gene expression analysis should be extended to cells of these stages.

One basic question can not be answered by this gene expression analysis, namely, whether the adult bone marrow is able to generate B1a cells under physiological conditions. To more definitely answer this question, a mouse model with inducible B cell development was established.

5.3. Generation of mice with inducible *Rag1* expression

The use of transgenic and gene targeting technology has been a great advantage for studying gene function in mice. However, in many cases transgene expression or gene inactivation results in embryonic lethality, such that only the earliest phenotype can be explored (Lobe and Nagy, 1998; Marth, 1996; Rajewsky *et al.*, 1996). A strategy to circumvent this limitation is the conditional control of gene expression (summarized in Lewandoski, 2001). Such control can be tissue-specific (spatial) and/or temporal. Thus, it is possible to silence or activate genes where and when desired as described above.

In the present work, conditional gene expression of *Rag1* was used to generate mice with inducible B cell development using ES cell technology. These newly generated mouse strains should, in addition to providing insights into antibody repertoire establishment and function of fetal liver derived B cells, definitively answer the question, whether the adult bone marrow also gives rise to B1a cells, and whether B1 and B2 cells are derived from different progenitors or not.

The frequency of homologous recombinants in ES cells in the present targeting experiments was astonishingly high. In situations in which the same genomic locus was targeted in multiple instances, generally a similar frequency of homologous recombination events has been observed. The targeting frequency from the generation of the *Rag1* knock-out mice (Mombaerts *et al.*, 1992) should therefore deliver a clue for the number of clones that had to be picked. In the study mentioned, the obtained frequencies were 1 out of 130 and 1 out of 117 targeted clones without the use of a negative selection marker.

After the transfection of the BALB/c and Bruce-4 ES cells only 60 clones for BALB/c and 24 clones for Bruce-4 were obtained. The lower number of the Bruce-4 derived clones was presumably due to the lower amount of vector DNA taken for transfection. Four clones of the BALB/c ES cells and two clones of the Bruce-4 ES cells showed homologous recombination. Thus targeting frequencies of 1 out of 15 and 1 out of 12 were obtained. Compared to the construct of Mombaerts, in the present work the arms of homology of the targeting vector used were substantially shorter, but negative selection was included in the procedure. However, the use of negative selection should lead to an enrichment of targeted clones of a factor 2 (Werner Müller, personal communication). One might speculate, that this significant enrichment was due to the second DTA cassette in the targeting vector, since in another targeting approach with a similar vector containing only one DTA cassette, no homologous recombinants out of 96 clones were found with Bruce-4 ES cells and 2 partial recombinants out of 96 clones with BALB/c ES cells (data not shown). On the other hand, this decrease could also be explained by the constitution of the particular ES cell preparation for the second transfection.

The targeting for the Indu-Rag1 mouse also led to partial homologous recombinants. This partial integration was likely caused by unfortunate features of the targeting vector, since the vector also contained regions of homology between the neomycin resistance cassette and the non-homologous part of the vector.

Before injection of the targeted ES cell clones into blastocysts, the recombination competence of the *loxP* sites was tested *in vitro*. Only the Cre concentration 0.25 µM gave rise to a recombination event. This does not imply that the recombination needs a specific concentration. This method of direct treatment is very rigorous and lead to the death of many cells. At higher Cre concentration many cells died. Only the lower Cre concentration left the cells alive and mediated the DNA recombination.

To obtain inducible B cell development, the inverted coding sequence of the *Rag1* gene was flanked by *loxP* sites in opposite orientation. Cre expression will lead to reversion and thus restoration of the normal transcription unit. However, continuous presence of recombinase in nuclei results in repeated inversions of the floxed DNA sequence. Constitutive presence of Cre or high expression levels might possibly cause genomic instability leading to deletions instead of inversions, as seen in Cre expressing *E. coli* (Aranda *et al.*, 2001).

To avoid such instability, Cre should only temporarily be expressed. To gain the temporal control over the recombination event, the Cre expression should be inducible. This induction can be obtained either at the transcriptional or posttranslational level. The Mx-Cre mice represent an example for induction at the transcriptional level (Kuhn *et al.*, 1995). In these mice a Cre recombinase transgene is controlled by the inducible promoter of the mouse *Mx1* gene. *Mx1* is part of the defense system against viral infections and is silent in healthy mice. The *Mx1* promoter can be transiently activated to high amounts of transcription in many tissues upon application of interferon α (IFN-α), IFN-β or of the IFN inducer synthetic double-stranded RNA (polyinosinic-polycytidylic acid, pI-pC; Staeheli *et al.*, 1986).

Induction at the posttranslational level employs fusion proteins of Cre with mutated ligand-binding domains of steroid receptors. In MerCreMer mice the Cre recombinase carries at both ends a mutated murine estrogen receptor (Mer) binding domain (Hobeika *et al.*, unpublished). The expression of this MerCreMer recombinase is controlled by the *mb-1* promoter that is specifically active in B cells. Therefore the MerCreMer mice represent, in contrast to the ubiquitous inducible Mx-Cre mice, an example for tissue-specific induction. Without induction, the MerCreMer fusion protein is in the cytoplasm in an inactive form. Induction with the estrogen analogue 4-hydroxytamoxifen (OHT) leads to the migration of the protein into the nucleus, where the DNA recombination event takes place. Both mice are available and will be used for further experiments.

It is still unclear if the induction with these Cre transgenic mice will lead to continuous lymphopoiesis or only to a wave of lymphocyte development. If long term HSCs are targeted, this should lead to continuous lymphopoiesis. If later developmental stages are targeted, this will only lead to short term reconstitution and a wave of lymphocyte development will be the result. In the case of the MerCreMer mice the latter situation is expected, since *mb-1* is an early B cell marker, indeed early but B cell specific.

An ambiguity in the employment of the Mx-Cre mice is their potential expression of Cre recombinase without induction due to endogenous production of IFN in these mice

('leakiness'). Background recombination was observed in some tissues, when crossing Mx-Cre mice to mice with floxed alleles (Kuhn *et al.*, 1995). These mice were maintained in a conventional facility. Thus, mice need to be maintained under specific pathogen free (SPF) conditions to lower endogenous IFN production and hence reduce the background recombination to negligible levels.

Recently, a new mouse strain has been described that expresses a fusion protein consisting of Cre recombinase and a mutated ligand-binding domain of the estrogen receptor. The coding region of the fusion protein was inserted into the ubiquitously expressed *Rosa26* locus through homologous recombination to allow induction by 4-hydroxytamoxifen administration in all organs. No background activity based on Southern blot analysis was observed in non-induced mice (Seibler *et al.*, 2003). This mouse appears as an ideal partner for the Indu-Rag1 mouse for the desired experiments.

Another possibility for the induction of the Indu-Rag1 mice is still under investigation. Protein transduction is a method to introduce biologically active proteins directly into mammalian cells with high efficiency. Up to now, the direct introduction of Cre recombinase was carried out *in vitro* and resulted in the inversion of *Rag1*.

One *in vivo* study reported transduction of Cre recombinase in mice, but recombination was not proven by Southern blot analysis (Jo *et al.*, 2001). Nevertheless modified Cre proteins could make it possible to directly treat the mice in the future. The newly generated Indu-Rag1 mice could hereby serve as an optimal test system. Already a low number of recombination events will lead to an accumulating number of B cells. These B cells will produce serum Ig, easy to measure by ELISA. The application of the Cre protein could be performed either intravenously or directly into the bone marrow.

A potential problem could be that only half of the cells will express *Rag1* after induction of Cre expression due to the nature of the recombination process. However, this should not lead to any problems in later experiments. Reconstitution of mice can be carried out with low numbers of HSCs. Such mice will still develop full immunological competence and normal lymphoid compartments.

In the future, a potential solution for this flipping characteristic of inverted *loxP* sites might be the use of mutated sites that mediate unidirectional genetic inversion in mice (Oberdoerffer *et al.*, 2003), thus obtaining a higher percentage of restored cells. One such *loxP* pair consists of two asymmetric, mutant *loxP* sites that carry a 5 bp mutation in the distal end of either the left (lox66) or the right (lox71) inverted repeat. Cre-mediated recombination of a lox66/lox71 pair

yields one *loxP* site with two mutated inverted repeats (lox72) and one wild-type *loxP* site. This pair showed a higher threshold for the initiation of Cre-mediated recombination than wild-type *loxP* sites and requires 2- to 3-fold higher Cre concentrations to recombine with an efficiency comparable to wild-type sites *in vitro*. Both *in vitro* and *in vivo*, no or decreased reverse recombination was observed with this system.

Besides the disadvantage, that only in 50% of the cells the *Rag1* gene can be activated, there is also the risk, that the second *loxP* site, placed in the 3'-UTR region could interfere with gene expression. The size of the genomic BamHI fragment, that ends shortly downstream the second exon of *Rag1*, and the limiting size of the targeting vector, prevented the possibility to flank the whole second exon with the *loxP* sites. In most targeting experiments, *loxP* sites were placed in introns, but sometimes have also been inserted in 5'- (Dragatsis *et al.*, 2000; Sakai *et al.*, 2001) or 3'-flanking regions (Guy *et al.*, 2001; Brakebusch *et al.*, 2000) without compromising gene expression. Thus this *loxP* site, most likely, should not disturb gene expression and function.

Another potential problem could be that the Indu-Rag1 mice might not be completely blocked in B and T cell development despite the inversion of the *Rag1* CDS. Although this possibility seems to be highly unlikely, the Indu-Rag1 mice should be compared with conventional $Rag1^{-/-}$ mice by flow cytometric analysis and measurement of serum Ig levels. After this confirmation it should be tested, if normal B cell development is influenced by the introduced *loxP* sites and the continuous inversion of the coding sequence during the presence of Cre in the nucleus. Therefore the Indu-Rag1(B6) mice should be crossed with mice that express the Cre recombinase constitutive under the control of the B cell specific CD19 promoter. This should allow B cell development in fetal liver as well as in the bone marrow. The emerging offspring should be compared – again by flow cytometry and measurement of serum Ig levels by ELSIA – with CD3 knock-out mice, that have also only B lymphocytes but no T lymphocytes.

After the establishment of an appropriate system for induction – Cre transgenic mice or purified Cre protein – the induction of B and T cell development, respectively, should be carried out at various time points after birth. The resulting mature B cell subpopulations should be analyzed by flow cytometric analysis. These experiments should then definitely answer the question whether the adult bone marrow gives rise to peritoneal B1a cells or not.

If B1a cells will be found in these mice, the antibody repertoire should be analyzed. In normal mice, B1a cells are especially generated in a phase, when TdT is not expressed. In addition,

the B1a compartments has been found to be enriched for certain B cell clones (Herzenberg *et al.*, 2000; Kretschmer *et al.*, 2002). This strong antigenic selection should work also in the presence of TdT in Indu-Rag1 mice. Thus, a similar bias might be observed.

The role of B1a cells as part of a 'first line of defense' against mucosal and systemic pathogens might be investigated in case of their absence in the adult induced Indu-Rag1 mice. The susceptibility of these mice against different pathogens should be investigated in comparison to normal wild-type mice. This investigation could be performed also even if B1a cells are developing in the adult induced mouse. This would demonstrate whether the fetal liver has an essential role in B cell ontogeny. It would extend experiments where enforced expression of TdT during fetal life in a transgenic mouse eliminated the well-characterized T15 anti-phosphorylcholine B cell subset that is protective against pneumococcal infection. These animals were susceptible to such infection (Benedict and Kearney, 1999). Similar experiments could be performed with the Indu-Rag1 mice.

By the inactivation of *Rag1* both lymphocyte compartments – B as well as T cells – are deleted. T cells however might be required for the complete development of the particular B cell compartments and/or some of their functions. To avoid such problems mice could be reconstituted with T cells or stem cells from mice that lack B cell development e.g. Ig$\alpha^{-/-}$ mice. Thus B cell development under both conditions can be compared.

For T cell development it was also suggested, that specific T cell subsets are only generated during fetal and neonatal development (V$\gamma5^{+}$ and V$\gamma6^{+}$ γ:δ T cells). This can now be confirmed in Indu-Rag1 mice. This new mouse model also enables to investigate potential functions of such subsets.

Taken together, a new mouse model on two different genetic backgrounds was established, that could be used to investigate different aspects of B cell development. It should deliver new insights in the different ontogeny between fetal liver and bone marrow derived B cell precursors. In addition, it can be used to investigate similar aspects of T cell development. This model can be used in the future for further approaches, where inducible lymphocyte development is required.

6. Summary

In the present work several aspects of B cell development were addressed. The first part focused on differential activation of variable regions of the light chain loci for the rearrangement process. Activation was monitored in a synchronously differentiating pro-B cell line by detection of germline transcripts. Several transcripts of V_κ families and all three transcripts of V_λ were cloned and characterized. Differential expression of V_κ germline transcripts from V_κ families proximal or distal to the JC_κ cluster was observed. Nevertheless, both clusters opened roughly at the same time as the JC_κ cluster. Similarly, V_λ and JC_λ opened almost synchronously. However compared to the κ locus, activation was found at a later developmental stage.

The second part concerned differences in B cell ontogeny between fetal liver and adult bone marrow. B cell precursors from either hematopoietic sites obviously give rise to distinct mature B cell subpopulations. Therefore, the gene expression patterns of early B cell precursors from fetal liver and adult bone marrow were compared using microarrays. This analysis confirmed previous findings and revealed further significant differences between both populations that can now be subjected to a more detailed analysis.

So far it is not possible to conclude from these results, whether such precursors represent committed progenitors for the different mature B cell subpopulations. As such, a mouse model was established, in which the origin of mature B cell subpopulations could be analyzed more definitely. In this model, the B cell development was made inducible. Therefore, the coding sequence of the *Rag1* gene was initially inverted for gene inactivation. Flanking of this region by *loxP* sites in opposite orientation renders it possible to revert this region by expression of Cre recombinase, thus restoring the transcription unit. This temporal control of Cre expression can be realized by the use of transgenic mice with inducible Cre expression thus allowing B cell development to be induced at discretion. Hence, the differentiation potential of bone marrow progenitors can be investigated after induction of B cell ontogeny in neonatal or adult mice. Since the *Rag1* gene was used for the targeting in this new mouse model, T cell development was rendered inducible in addition. Therefore, this mouse strain could be used to answer similar questions in T cell ontogeny.

7. References

Ahearn, J.M., Fischer, M.B., Croix, D., Goerg, S., Ma, M., Xia, J., Zhou, X., Howard, R.G., Rothstein, T.L., and Carroll, M.C. (1996). Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity. *4*, 251-262.

Akashi, K., Reya, T., Dalma-Weiszhausz, D., and Weissman, I.L. (2000a). Lymphoid precursors. Curr. Opin. Immunol. *12*, 144-150.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000b). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Alessandrini, A. and Desiderio, S.V. (1991). Coordination of immunoglobulin DJH transcription and D-to-JH rearrangement by promoter-enhancer approximation. Mol. Cell Biol. *11*, 2096-2107.

Allman, D., Li, J., and Hardy, R.R. (1999). Commitment to the B lymphoid lineage occurs before DH-JH recombination. J. Exp. Med. *189*, 735-740.

Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A., and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. Nat. Immunol. *4*, 168-174.

Alt, F.W., Blackwell, T.K., and Yancopoulos, G.D. (1987). Development of the primary antibody repertoire. Science *238*, 1079-1087.

Alt, F.W., Rosenberg, N., Enea, V., Siden, E., and Baltimore, D. (1982). Multiple immunoglobulin heavy-chain gene transcripts in Abelson murine leukemia virus-transformed lymphoid cell lines. Mol. Cell Biol. *2*, 386-400.

Aranda, M., Kanellopoulou, C., Christ, N., Peitz, M., Rajewsky, K., and Droge, P. (2001). Altered directionality in the Cre-LoxP site-specific recombination pathway. J. Mol. Biol. *311*, 453-459.

Arney, K.L. (2003). H19 and Igf2--enhancing the confusion? Trends Genet. *19*, 17-23.

Arnold, L.W., Pennell, C.A., McCray, S.K., and Clarke, S.H. (1994). Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. J. Exp. Med. *179*, 1585-1595.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Avila, E.M., Holdsworth, G., Sasaki, N., Jackson, R.L., and Harmony, J.A. (1982). Apoprotein E suppresses phytohemagglutinin-activated phospholipid turnover in peripheral blood mononuclear cells. J. Biol. Chem. *257*, 5900-5909.

Balazs, M., Martin, F., Zhou, T., and Kearney, J. (2002). Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity. *17*, 341-352.

Barnes, D.E., Stamp, G., Rosewell, I., Denzel, A., and Lindahl, T. (1998). Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. Curr. Biol. *8*, 1395-1398.

Baron, U. and Bujard, H. (2000). Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. Methods Enzymol. *327*, 401-421.

Barreto, V., Marques, R., and Demengeot, J. (2001). Early death and severe lymphopenia caused by ubiquitous expression of the Rag1 and Rag2 genes in mice. Eur. J. Immunol. *31*, 3763-3772.

Baumgarth, N. (2000). A two-phase model of B-cell activation. Immunol. Rev. *176*, 171-180.

Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L.E., Herzenberg, L.A., and Chen, J. (2000). B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J. Exp. Med. *192*, 271-280.

Bemark, M., Liberg, D., and Leanderson, T. (1998). Conserved sequence elements in K promoters from mice and humans: implications for transcriptional regulation and repertoire expression. Immunogenetics *47*, 183-195.

Bendelac, A., Bonneville, M., and Kearney, J.F. (2001). Autoreactivity by design: innate B and T lymphocytes. Nat. Rev. Immunol. *1*, 177-186.

Benedict, C.L., Gilfillan, S., and Kearney, J.F. (2001). The long isoform of terminal deoxynucleotidyl transferase enters the nucleus and, rather than catalyzing nontemplated nucleotide addition, modulates the catalytic activity of the short isoform. J. Exp. Med. *193*, 89-99.

Benedict, C.L. and Kearney, J.F. (1999). Increased junctional diversity in fetal B cells results in a loss of protective anti-phosphorylcholine antibodies in adult mice. Immunity. *10*, 607-617.

Bentolila, L.A., Wu, G.E., Nourrit, F., Fanton, d.M., Rougeon, F., and Doyen, N. (1997). Constitutive expression of terminal deoxynucleotidyl transferase in transgenic mice is sufficient for N region diversity to occur at any Ig locus throughout B cell differentiation. J. Immunol. *158*, 715-723.

Berland, R. and Wortis, H.H. (2002). Origins and functions of B-1 cells with notes on the role of CD5. Annu. Rev. Immunol. *20*, 253-300.

Bertrand, F.E., Eckfeldt, C.E., Lysholm, A.S., and LeBien, T.W. (2000). Notch-1 and Notch-2 exhibit unique patterns of expression in human B-lineage cells. Leukemia *14*, 2095-2102.

Bertrand, F.E., III, Olson, S.L., Martin, D.A., and Wu, G.E. (1998). Sequence analysis of the mouse RAG locus intergenic region. Dev. Immunol. *5*, 215-222.

Besmer, E., Gourzi, P., and Papavasiliou, F.N. (2004). The regulation of somatic hypermutation. Curr. Opin. Immunol. *16*, 241-245.

Besmer, E., Mansilla-Soto, J., Cassard, S., Sawchuk, D.J., Brown, G., Sadofsky, M., Lewis, S.M., Nussenzweig, M.C., and Cortes, P. (1998). Hairpin coding end opening is mediated by RAG1 and RAG2 proteins. Mol. Cell *2*, 817-828.

Blackwell, T.K., Moore, M.W., Yancopoulos, G.D., Suh, H., Lutzker, S., Selsing, E., and Alt, F.W. (1986). Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature *324*, 585-589.

Blomberg, B. and Tonegawa, S. (1982). DNA sequences of the joining regions of mouse lambda light chain immunoglobulin genes. Proc. Natl. Acad. Sci. U. S. A *79*, 530-533.

Boes, M., Prodeus, A.P., Schmidt, T., Carroll, M.C., and Chen, J. (1998). A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. *188*, 2381-2386.

Born, W., Cady, C., Jones-Carson, J., Mukasa, A., Lahn, M., and O'Brien, R. (1999). Immunoregulatory functions of gamma delta T cells. Adv. Immunol. *71*, 77-144.

Bosma, M.J. and Carroll, A.M. (1991). The SCID mouse mutant: definition, characterization, and potential uses. Annu. Rev. Immunol. *9*, 323-350.

Brakebusch, C., Grose, R., Quondamatteo, F., Ramirez, A., Jorcano, J.L., Pirro, A., Svensson, M., Herken, R., Sasaki, T., Timpl, R., Werner, S., and Fassler, R. (2000). Skin and hair follicle integrity is crucially dependent on beta 1 integrin expression on keratinocytes. EMBO J. *19*, 3990-4003.

Brandtzaeg, P. (1989). Overview of the mucosal immune system. Curr. Top. Microbiol. Immunol. *146*, 13-25.

Brannan, C.I., Dees, E.C., Ingram, R.S., and Tilghman, S.M. (1990). The product of the H19 gene may function as an RNA. Mol. Cell Biol. *10*, 28-36.

Brasel, K., McKenna, H.J., Morrissey, P.J., Charrier, K., Morris, A.E., Lee, C.C., Williams, D.E., and Lyman, S.D. (1996). Hematologic effects of flt3 ligand in vivo in mice. Blood *88*, 2004-2012.

Briles, D.E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J., and Barletta, R. (1981). Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. J. Exp. Med. *153*, 694-705.

Bubien, J.K., Zhou, L.J., Bell, P.D., Frizzell, R.A., and Tedder, T.F. (1993). Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. J. Cell Biol. *121*, 1121-1132.

Buchholz, F., Angrand, P.O., and Stewart, A.F. (1996a). A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. Nucleic Acids Res. *24*, 3118-3119.

Buchholz, F., Angrand, P.O., and Stewart, A.F. (1998). Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat. Biotechnol. *16*, 657-662.

Buchholz, F., Ringrose, L., Angrand, P.O., Rossi, F., and Stewart, A.F. (1996b). Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. Nucleic Acids Res. *24*, 4256-4262.

Bulla, R., Bossi, F., Radillo, O., de Seta, F., and Tedesco, F. (2003). Placental trophoblast and endothelial cells as target of maternal immune response. Autoimmunity *36*, 11-18.

Burkly, L.C., Jakubowski, A., Newman, B.M., Rosa, M.D., Chi-Rosso, G., and Lobb, R.R. (1991). Signaling by vascular cell adhesion molecule-1 (VCAM-1) through VLA-4 promotes CD3-dependent T cell proliferation. Eur. J. Immunol. *21*, 2871-2875.

Busslinger, M., Nutt, S.L., and Rolink, A.G. (2000). Lineage commitment in lymphopoiesis. Curr. Opin. Immunol. *12*, 151-158.

Cao, T., Longley, M.A., Wang, X.J., and Roop, D.R. (2001). An inducible mouse model for epidermolysis bullosa simplex: implications for gene therapy. J. Cell Biol. *152*, 651-656.

Carlsson, L. and Holmberg, D. (1990). Genetic basis of the neonatal antibody repertoire: germline V-gene expression and limited N-region diversity. Int. Immunol. *2*, 639-643.

Carmack, C.E., Shinton, S.A., Hayakawa, K., and Hardy, R.R. (1990). Rearrangement and selection of VH11 in the Ly-1 B cell lineage. J. Exp. Med. *172*, 371-374.

Carvalho, T.L., Mota-Santos, T., Cumano, A., Demengeot, J., and Vieira, P. (2001). Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. J. Exp. Med. *194*, 1141-1150.

Casali, P. and Schettino, E.W. (1996). Structure and function of natural antibodies. Curr. Top. Microbiol. Immunol. *210*, 167-179.

Casellas, R., Jankovic, M., Meyer, G., Gazumyan, A., Luo, Y., Roeder, R., and Nussenzweig, M. (2002). OcaB is required for normal transcription and V(D)J recombination of a subset of immunoglobulin kappa genes. Cell *110*, 575-585.

Chan, V.W., Meng, F., Soriano, P., DeFranco, A.L., and Lowell, C.A. (1997). Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity. *7*, 69-81.

Chefalo, P.J., Grandea, A.G., III, Van Kaer, L., and Harding, C.V. (2003). Tapasin-/- and TAP1-/- macrophages are deficient in vacuolar alternate class I MHC (MHC-I) processing due to decreased MHC-I stability at phagolysosomal pH. J. Immunol. *170*, 5825-5833.

Chen, J., Trounstine, M., Kurahara, C., Young, F., Kuo, C.C., Xu, Y., Loring, J.F., Alt, F.W., and Huszar, D. (1993a). B cell development in mice that lack one or both immunoglobulin kappa light chain genes. EMBO J. *12*, 821-830.

Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R.K., and Alt, F.W. (1993b). Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. EMBO J. *12*, 4635-4645.

Cherrier, M., Cardona, A., Rosinski-Chupin, I., Rougeon, F., and Doyen, N. (2002). Substantial N diversity is generated in T cell receptor alpha genes at birth despite low levels of terminal deoxynucleotidyl transferase expression in mouse thymus. Eur. J. Immunol. *32*, 3651-3656.

Cherry, S.R. and Baltimore, D. (1999). Chromatin remodeling directly activates V(D)J recombination. Proc. Natl. Acad. Sci. U. S. A *96*, 10788-10793.

Cherry, S.R., Beard, C., Jaenisch, R., and Baltimore, D. (2000). V(D)J recombination is not activated by demethylation of the kappa locus. Proc. Natl. Acad. Sci. U. S. A *97*, 8467-8472.

Chukwuocha, R.U., Nadel, B., and Feeney, A.J. (1995). Analysis of homology-directed recombination in VDJ junctions from cytoplasmic Ig- pre-B cells of newborn mice. J. Immunol. *154*, 1246-1255.

Chumley, M.J., Dal Porto, J.M., Kawaguchi, S., Cambier, J.C., Nemazee, D., and Hardy, R.R. (2000). A VH11V kappa 9 B cell antigen receptor drives generation of CD5+ B cells both in vivo and in vitro. J. Immunol. *164*, 4586-4593.

Chun, J.J., Schatz, D.G., Oettinger, M.A., Jaenisch, R., and Baltimore, D. (1991). The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell *64*, 189-200.

Classon, B.J. and Coverdale, L. (1994). Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins. Proc. Natl. Acad. Sci. U. S. A *91*, 5296-5300.

Cocea, L., De Smet, A., Saghatchian, M., Fillatreau, S., Ferradini, L., Schurmans, S., Weill, J.C., and Reynaud, C.A. (1999). A targeted deletion of a region upstream from the Jkappa cluster impairs kappa chain rearrangement in cis in mice and in the 103/bcl2 cell line. J. Exp. Med. *189*, 1443-1450.

Cogne, M., Lansford, R., Bottaro, A., Zhang, J., Gorman, J., Young, F., Cheng, H.L., and Alt, F.W. (1994). A class switch control region at the 3' end of the immunoglobulin heavy chain locus. Cell *77*, 737-747.

Cong, Y.Z., Rabin, E., and Wortis, H.H. (1991). Treatment of murine CD5- B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. Int. Immunol. *3*, 467-476.

Constancia, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C., and Reik, W. (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature *417*, 945-948.

Cotta, C.V., Zhang, Z., Kim, H.G., and Klug, C.A. (2003). Pax5 determines B- versus T-cell fate and does not block early myeloid-lineage development. Blood *101*, 4342-4346.

Coutinho, A., Kazatchkine, M.D., and Avrameas, S. (1995). Natural autoantibodies. Curr. Opin. Immunol. *7*, 812-818.

Critchlow, S.E., Bowater, R.P., and Jackson, S.P. (1997). Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. Curr. Biol. *7*, 588-598.

Crompton, T., Outram, S.V., Buckland, J., and Owen, M.J. (1998). Distinct roles of the interleukin-7 receptor alpha chain in fetal and adult thymocyte development revealed by analysis of interleukin-7 receptor alpha-deficient mice. Eur. J. Immunol. *28*, 1859-1866.

Cumano, A., Paige, C.J., Iscove, N.N., and Brady, G. (1992). Bipotential precursors of B cells and macrophages in murine fetal liver. Nature *356*, 612-615.

Daitch, L.E., Moore, M.W., Persiani, D.M., Durdik, J.M., and Selsing, E. (1992). Transcription and recombination of the murine RS element. J. Immunol. *149*, 832-840.

de Bont, N., Netea, M.G., Demacker, P.N., Verschueren, I., Kullberg, B.J., van Dijk, K.W., van der Meer, J.W., and Stalenhoef, A.F. (1999). Apolipoprotein E knock-out mice are highly susceptible to endotoxemia and Klebsiella pneumoniae infection. J. Lipid Res. *40*, 680-685.

DeKoter, R.P., Lee, H.J., and Singh, H. (2002). PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. Immunity. *16*, 297-309.

DeKoter, R.P. and Singh, H. (2000). Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science *288*, 1439-1441.

Doody, G.M., Bell, S.E., Vigorito, E., Clayton, E., McAdam, S., Tooze, R., Fernandez, C., Lee, I.J., and Turner, M. (2001). Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. Nat. Immunol. *2*, 542-547.

Douagi, I., Vieira, P., and Cumano, A. (2002). Lymphocyte commitment during embryonic development, in the mouse. Semin. Immunol. *14*, 361-369.

Dougherty, G.J., Kay, R.J., and Humphries, R.K. (1989). Molecular cloning of 114/A10, a cell surface antigen containing highly conserved repeated elements, which is expressed by murine hemopoietic progenitor cells and interleukin-3-dependent cell lines. J. Biol. Chem. *264*, 6509-6514.

Doyen, N., d'Andon, M.F., Bentolila, L.A., Nguyen, Q.T., and Rougeon, F. (1993). Differential splicing in mouse thymus generates two forms of terminal deoxynucleotidyl transferase. Nucleic Acids Res. *21*, 1187-1191.

Dragatsis, I., Levine, M.S., and Zeitlin, S. (2000). Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat. Genet. *26*, 300-306.

Dunda, O. and Corcos, D. (1997). Recombining sequence recombination in normal kappa-chain-expressing B cells. J. Immunol. *159*, 4362-4366.

Durdik, J., Moore, M.W., and Selsing, E. (1984). Novel kappa light-chain gene rearrangements in mouse lambda light chain-producing B lymphocytes. Nature *307*, 749-752.

Dymecki, S.M. (1996). Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc. Natl. Acad. Sci. U. S. A *93*, 6191-6196.

Egan, S.E., St Pierre, B., and Leow, C.C. (1998). Notch receptors, partners and regulators: from conserved domains to powerful functions. Curr. Top. Microbiol. Immunol. *228*, 273-324.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell *66*, 649-661.

Engel, H., Bogen, B., Muller, U., Andersson, J., Rolink, A., and Weiss, S. (1998). Expression level of a transgenic lambda2 chain results in isotype exclusion and commitment to B1 cells. Eur. J. Immunol. *28*, 2289-2299.

Engel, H., Rolink, A., and Weiss, S. (1999). B cells are programmed to activate kappa and lambda for rearrangement at consecutive developmental stages. Eur. J. Immunol. *29*, 2167-2176.

Engel, H., Ruhl, H., Benham, C.J., Bode, J., and Weiss, S. (2001). Germ-line transcripts of the immunoglobulin lambda J-C clusters in the mouse: characterization of the initiation sites and regulatory elements. Mol. Immunol. *38*, 289-302.

Engel, P., Zhou, L.J., Ord, D.C., Sato, S., Koller, B., and Tedder, T.F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. *3*, 39-50.

Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K., and Honjo, T. (2001). In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature *413*, 639-643.

Fagarasan, S., Shinkura, R., Kamata, T., Nogaki, F., Ikuta, K., Tashiro, K., and Honjo, T. (2000). Alymphoplasia (aly)-type nuclear factor kappaB-inducing kinase (NIK) causes defects in secondary lymphoid tissue chemokine receptor signaling and homing of peritoneal cells to the gut-associated lymphatic tissue system. J. Exp. Med. *191*, 1477-1486.

Feeney, A.J. (1990). Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J. Exp. Med. *172*, 1377-1390.

Feeney, A.J. (1992). Predominance of VH-D-JH junctions occurring at sites of short sequence homology results in limited junctional diversity in neonatal antibodies. J. Immunol. *149*, 222-229.

Feeney, A.J., Victor, K.D., Vu, K., Nadel, B., and Chukwuocha, R.U. (1994). Influence of the V(D)J recombination mechanism on the formation of the primary T and B cell repertoires. Semin. Immunol. *6*, 155-163.

Fondell, J.D. and Marcu, K.B. (1992). Transcription of germ line V alpha segments correlates with ongoing T-cell receptor alpha-chain rearrangement. Mol. Cell Biol. *12*, 1480-1489.

Forster, I. and Rajewsky, K. (1990). The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. Proc. Natl. Acad. Sci. U. S. A *87*, 4781-4784.

Frank, K.M., Sekiguchi, J.M., Seidl, K.J., Swat, W., Rathbun, G.A., Cheng, H.L., Davidson, L., Kangaloo, L., and Alt, F.W. (1998). Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. Nature *396*, 173-177.

Frazer, J.K. and Capra, J.D. (1999). Immunoglobulins: Structure and Function. In *Fundamental immunology* (Edited by Paul W.E.), pp. 37-74, Lippincott-Raven, Philadelphia.

Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J. Exp. Med. *181*, 1519-1526.

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W., and Cantley, L.C. (1999). Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science *283*, 393-397.

Fugmann, S.D., Lee, A.I., Shockett, P.E., Villey, I.J., and Schatz, D.G. (2000). The RAG proteins and V(D)J recombination: complexes, ends, and transposition. Annu. Rev. Immunol. *18*, 495-527.

Fuller, K. and Storb, U. (1997). Identification and characterization of the murine Rag1 promoter. Mol. Immunol. *34*, 939-954.

Gao, Y., Chaudhuri, J., Zhu, C., Davidson, L., Weaver, D.T., and Alt, F.W. (1998a). A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. Immunity. *9*, 367-376.

Gao, Y., Sun, Y., Frank, K.M., Dikkes, P., Fujiwara, Y., Seidl, K.J., Sekiguchi, J.M., Rathbun, G.A., Swat, W., Wang, J., Bronson, R.T., Malynn, B.A., Bryans, M., Zhu, C., Chaudhuri, J., Davidson, L., Ferrini, R., Stamato, T., Orkin, S.H., Greenberg, M.E., and Alt, F.W. (1998b). A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell *95*, 891-902.

Garbi, N., Tiwari, N., Momburg, F., and Hammerling, G.J. (2003). A major role for tapasin as a stabilizer of the TAP peptide transporter and consequences for MHC class I expression. Eur. J. Immunol. *33*, 264-273.

Gellert, M. (1997). Recent advances in understanding V(D)J recombination. Adv. Immunol. *64*, 39-64.

Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G., and Maiese, K. (1997). Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol. Rev. *159*, 95-103.

Gerstein, R.M. and Lieber, M.R. (1993). Extent to which homology can constrain coding exon junctional diversity in V(D)J recombination. Nature *363*, 625-627.

Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C., and Mathis, D. (1993). Mice lacking TdT: mature animals with an immature lymphocyte repertoire. Science *261*, 1175-1178.

Godin, I.E., Garcia-Porrero, J.A., Coutinho, A., Dieterlen-Lievre, F., and Marcos, M.A. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature *364*, 67-70.

Golding, A., Chandler, S., Ballestar, E., Wolffe, A.P., and Schlissel, M.S. (1999). Nucleosome structure completely inhibits in vitro cleavage by the V(D)J recombinase. EMBO J. *18*, 3712-3723.

Goldman, J.P., Spencer, D.M., and Raulet, D.H. (1993). Ordered rearrangement of variable region genes of the T cell receptor gamma locus correlates with transcription of the unrearranged genes. J. Exp. Med. *177*, 729-739.

Gong, S. and Nussenzweig, M.C. (1996). Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. Science *272*, 411-414.

Goodhardt, M., Cavelier, P., Doyen, N., Kallenbach, S., Babinet, C., and Rougeon, F. (1993). Methylation status of immunoglobulin kappa gene segments correlates with their recombination potential. Eur. J. Immunol. *23*, 1789-1795.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., and . (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676-682.

Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, S.K., March, C.J., Namen, A.E., and . (1990). Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell *60*, 941-951.

Gorman, J.R., van der, S.N., Monroe, R., Cogne, M., Davidson, L., and Alt, F.W. (1996). The Ig(kappa) enhancer influences the ratio of Ig(kappa) versus Ig(lambda) B lymphocytes. Immunity. *5*, 241-252.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U. S. A *89*, 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766-1769.

Grawunder, U., Haasner, D., Melchers, F., and Rolink, A. (1993). Rearrangement and expression of kappa light chain genes can occur without mu heavy chain expression during differentiation of pre-B cells. Int. Immunol. *5*, 1609-1618.

Grawunder, U., Leu, T.M., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F., and Winkler, T.H. (1995a). Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity. *3*, 601-608.

Grawunder, U., Rolink, A., and Melchers, F. (1995b). Induction of sterile transcription from the kappa L chain gene locus in V(D)J recombinase-deficient progenitor B cells. Int. Immunol. *7*, 1915-1925.

Grawunder, U., Schatz, D.G., Leu, T.M., Rolink, A., and Melchers, F. (1996). The half-life of RAG-1 protein in precursor B cells is increased in the absence of RAG-2 expression. J. Exp. Med. *183*, 1731-1737.

Grawunder, U., West, R.B., and Lieber, M.R. (1998). Antigen receptor gene rearrangement. Curr. Opin. Immunol. *10*, 172-180.

Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T.E., Mann, M., and Lieber, M.R. (1997). Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. Nature *388*, 492-495.

Gray, D., MacLennan, I.C., Bazin, H., and Khan, M. (1982). Migrant mu+ delta+ and static mu+ delta- B lymphocyte subsets. Eur. J. Immunol. *12*, 564-569.

Greenbaum, S. and Zhuang, Y. (2002). Regulation of early lymphocyte development by E2A family proteins. Semin. Immunol. *14*, 405-414.

Gu, H., Forster, I., and Rajewsky, K. (1990). Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J. *9*, 2133-2140.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science *265*, 103-106.

Gu, Y., Jin, S., Gao, Y., Weaver, D.T., and Alt, F.W. (1997). Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. Proc. Natl. Acad. Sci. U. S. A *94*, 8076-8081.

Guy, J., Hendrich, B., Holmes, M., Martin, J.E., and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat. Genet. *27*, 322-326.

Hagman, J., Rudin, C.M., Haasch, D., Chaplin, D., and Storb, U. (1990). A novel enhancer in the immunoglobulin lambda locus is duplicated and functionally independent of NF kappa B. Genes Dev. *4*, 978-992.

Haines, B.B. and Brodeur, P.H. (1998). Accessibility changes across the mouse Igh-V locus during B cell development. Eur. J. Immunol. *28*, 4228-4235.

Hamilton, A.M. and Kearney, J.F. (1994). Effects of IgM allotype suppression on serum IgM levels, B-1 and B-2 cells, and antibody responses in allotype heterozygous F1 mice. Dev. Immunol. *4*, 27-41.

Han, S., Zheng, B., Schatz, D.G., Spanopoulou, E., and Kelsoe, G. (1996). Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. Science *274*, 2094-2097.

Hansen, J.D. and Kaattari, S.L. (1995). The recombination activation gene 1 (RAG1) of rainbow trout (Oncorhynchus mykiss): cloning, expression, and phylogenetic analysis. Immunogenetics *42*, 188-195.

Hao, Z. and Rajewsky, K. (2001). Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J. Exp. Med. *194*, 1151-1164.

Hardy, R.R. (2003). B-cell commitment: deciding on the players. Curr. Opin. Immunol. *15*, 158-165.

Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D., and Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. *173*, 1213-1225.

Hardy, R.R., Carmack, C.E., Shinton, S.A., Riblet, R.J., and Hayakawa, K. (1989). A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. J. Immunol. *142*, 3643-3651.

Hardy, R.R., Wasserman, R., Li, Y.S., Shinton, S.A., and Hayakawa, K. (2000). Response by B cell precursors to pre-B receptor assembly: differences between fetal liver and bone marrow. Curr. Top. Microbiol. Immunol. *252*, 25-30.

Hartley, S.B., Cooke, M.P., Fulcher, D.A., Harris, A.W., Cory, S., Basten, A., and Goodnow, C.C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell *72*, 325-335.

Hashimoto, A., Takeda, K., Inaba, M., Sekimata, M., Kaisho, T., Ikehara, S., Homma, Y., Akira, S., and Kurosaki, T. (2000). Cutting edge: essential role of phospholipase C-gamma 2 in B cell development and function. J. Immunol. *165*, 1738-1742.

Hasserjian, R.P., Aster, J.C., Davi, F., Weinberg, D.S., and Sklar, J. (1996). Modulated expression of notch1 during thymocyte development. Blood *88*, 970-976.

Havran, W.L. and Boismenu, R. (1994). Activation and function of gamma delta T cells. Curr. Opin. Immunol. *6*, 442-446.

Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Allman, D., Stewart, C.L., Silver, J., and Hardy, R.R. (1999). Positive selection of natural autoreactive B cells. Science *285*, 113-116.

Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Wen, L.J., Dashoff, J., and Hardy, R.R. (2003). Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. J. Exp. Med. *197*, 87-99.

Hayakawa, K. and Hardy, R.R. (2000). Development and function of B-1 cells. Curr. Opin. Immunol. *12*, 346-353.

Hayakawa, K., Hardy, R.R., Herzenberg, L.A., and Herzenberg, L.A. (1985). Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J. Exp. Med. *161*, 1554-1568.

Hayakawa, K., Hardy, R.R., Stall, A.M., Herzenberg, L.A., and Herzenberg, L.A. (1986). Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. Eur. J. Immunol. *16*, 1313-1316.

Hayakawa, K., Tarlinton, D., and Hardy, R.R. (1994). Absence of MHC class II expression distinguishes fetal from adult B lymphopoiesis in mice. J. Immunol. *152*, 4801-4807.

Hayakawa, S., Tochigi, M., Chishima, F., Shiraishi, H., Takahashi, N., Watanabe, K., Fujii, K.T., and Satoh, K. (1996). Expression of the recombinase-activating gene (RAG-1) in murine early embryogenesis. Immunol. Cell Biol. *74*, 52-56.

Hayhurst, G.P., Lee, Y.H., Lambert, G., Ward, J.M., and Gonzalez, F.J. (2001). Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol. Cell Biol. *21*, 1393-1403.

Hein, K., Lorenz, M.G., Siebenkotten, G., Petry, K., Christine, R., and Radbruch, A. (1998). Processing of switch transcripts is required for targeting of antibody class switch recombination. J. Exp. Med. *188*, 2369-2374.

Hendriks, R.W., de Bruijn, M.F., Maas, A., Dingjan, G.M., Karis, A., and Grosveld, F. (1996). Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. EMBO J. *15*, 4862-4872.

Herzenberg, L.A. (2000). B-1 cells: the lineage question revisited. Immunol. Rev. *175*, 9-22.

Herzenberg, L.A., Baumgarth, N., and Wilshire, J.A. (2000). B-1 cell origins and VH repertoire determination. Curr. Top. Microbiol. Immunol. *252*, 3-13.

Hikida, M., Mori, M., Takai, T., Tomochika, K., Hamatani, K., and Ohmori, H. (1996). Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. Science *274*, 2092-2094.

Hikida, M. and Ohmori, H. (1998). Rearrangement of lambda light chain genes in mature B cells in vitro and in vivo. Function of reexpressed recombination-activating gene (RAG) products. J. Exp. Med. *187*, 795-799.

Holman, P.O., Roth, M.E., Huang, M., and Kranz, D.M. (1993). Characterization of transcripts from unrearranged V alpha 8 genes in the thymus. J. Immunol. *151*, 1959-1967.

Hood, L., Gray, W.R., Sanders, B.G., and Dreyer, W.J. (1967). Light chain evolution. Cold Spring Harb. Symp. Quant. Biol. *32*, 133-146.

Hsieh, C.S., deRoos, P., Honey, K., Beers, C., and Rudensky, A.Y. (2002). A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. J. Immunol. *168*, 2618-2625.

Hsu, L.Y., Lauring, J., Liang, H.E., Greenbaum, S., Cado, D., Zhuang, Y., and Schlissel, M.S. (2003). A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. Immunity. *19*, 105-117.

Humbert, P.O. and Corcoran, L.M. (1997). oct-2 gene disruption eliminates the peritoneal B-1 lymphocyte lineage and attenuates B-2 cell maturation and function. J. Immunol. *159*, 5273-5284.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y.H., and Weissman, I.L. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. Cell *62*, 863-874.

Itohara, S., Nakanishi, N., Kanagawa, O., Kubo, R., and Tonegawa, S. (1989). Monoclonal antibodies specific to native murine T-cell receptor gamma delta: analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. Proc. Natl. Acad. Sci. U. S. A *86*, 5094-5098.

Izon, D., Rudd, K., DeMuth, W., Pear, W.S., Clendenin, C., Lindsley, R.C., and Allman, D. (2001). A common pathway for dendritic cell and early B cell development. J. Immunol. *167*, 1387-1392.

Jo, D., Nashabi, A., Doxsee, C., Lin, Q., Unutmaz, D., Chen, J., and Ruley, H.E. (2001). Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase. Nat. Biotechnol. *19*, 929-933.

Johansen, F.E., Braathen, R., and Brandtzaeg, P. (2000). Role of J chain in secretory immunoglobulin formation. Scand. J. Immunol. *52*, 240-248.

Johnson, K. and Calame, K. (2003). Transcription factors controlling the beginning and end of B-cell differentiation. Curr. Opin. Genet. Dev. *13*, 522-528.

Jones, P., May, G., Healy, L., Brown, J., Hoyne, G., Delassus, S., and Enver, T. (1998). Stromal expression of Jagged 1 promotes colony formation by fetal hematopoietic progenitor cells. Blood *92*, 1505-1511.

Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M., and Nielsen, P.J. (1999). Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. Immunity. *11*, 547-554.

Jurka, J., Klonowski, P., Dagman, V., and Pelton, P. (1996). CENSOR--a program for identification and elimination of repetitive elements from DNA sequences. Comput. Chem. *20*, 119-121.

Kantor, A.B. and Herzenberg, L.A. (1993). Origin of murine B cell lineages. Annu. Rev. Immunol. *11*, 501-538.

Kantor, A.B., Merrill, C.E., Herzenberg, L.A., and Hillson, J.L. (1997). An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. J. Immunol. *158*, 1175-1186.

Kantor, A.B., Merrill, C.E., MacKenzie, J.D., Herzenberg, L.A., and Hillson, J.L. (1995a). Development of the antibody repertoire as revealed by single-cell PCR of FACS-sorted B-cell subsets. Ann. N. Y. Acad. Sci. *764*, 224-227.

Kantor, A.B., Stall, A.M., Adams, S., Herzenberg, L.A., and Herzenberg, L.A. (1992). Differential development of progenitor activity for three B-cell lineages. Proc. Natl. Acad. Sci. U. S. A *89*, 3320-3324.

Kantor, A.B., Stall, A.M., Adams, S., Watanabe, K., and Herzenberg, L.A. (1995b). De novo development and self-replenishment of B cells. Int. Immunol. *7*, 55-68.

Kanzaki, M., Shibata, H., Mogami, H., and Kojima, I. (1995). Expression of calcium-permeable cation channel CD20 accelerates progression through the G1 phase in Balb/c 3T3 cells. J. Biol. Chem. *270*, 13099-13104.

Karre, K. (2002). NK cells, MHC class I molecules and the missing self. Scand. J. Immunol. *55*, 221-228.

Kawamoto, H., Ikawa, T., Ohmura, K., Fujimoto, S., and Katsura, Y. (2000). T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. Immunity. *12*, 441-450.

Kelley, D.E., Pollok, B.A., Atchison, M.L., and Perry, R.P. (1988). The coupling between enhancer activity and hypomethylation of kappa immunoglobulin genes is developmentally regulated. Mol. Cell Biol. *8*, 930-937.

Kemp, D.J., Harris, A.W., Cory, S., and Adams, J.M. (1980). Expression of the immunoglobulin C mu gene in mouse T and B lymphoid and myeloid cell lines. Proc. Natl. Acad. Sci. U. S. A *77*, 2876-2880.

Kenter, A.L. and Tredup, J. (1991). High expression of a 3'----5' exonuclease activity is specific to B lymphocytes. Mol. Cell Biol. *11*, 4398-4404.

Keyna, U., Beck-Engeser, G.B., Jongstra, J., Applequist, S.E., and Jack, H.M. (1995). Surrogate light chain-dependent selection of Ig heavy chain V regions. J. Immunol. *155*, 5536-5542.

Khan, W.N., Alt, F.W., Gerstein, R.M., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A.B., Herzenberg, L.A., and . (1995). Defective B cell development and function in Btk-deficient mice. Immunity. *3*, 283-299.

Khanna, K.K. and Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat. Genet. *27*, 247-254.

Kim, K.M., Alber, G., Weiser, P., and Reth, M. (1993). Signalling function of the B-cell antigen receptors. Immunol. Rev. *132*, 125-146.

Kincade, P.W., Owen, J.J., Igarashi, H., Kouro, T., Yokota, T., and Rossi, M.I. (2002). Nature or nurture? Steady-state lymphocyte formation in adults does not recapitulate ontogeny. Immunol. Rev. *187*, 116-125.

Kirschbaum, T., Pourrajabi, S., Zocher, I., Schwendinger, J., Heim, V., Roschenthaler, F., Kirschbaum, V., and Zachau, H.G. (1998). The 3' part of the immunoglobulin kappa locus of the mouse. Eur. J. Immunol. *28*, 1458-1466.

Kirschbaum, T., Roschenthaler, F., Bensch, A., Holscher, B., Lautner-Rieske, A., Ohnrich, M., Pourrajabi, S., Schwendinger, J., Zocher, I., and Zachau, H.G. (1999). The central part of the mouse immunoglobulin kappa locus. Eur. J. Immunol. *29*, 2057-2064.

Kitamura, D., Kudo, A., Schaal, S., Muller, W., Melchers, F., and Rajewsky, K. (1992). A critical role of lambda 5 protein in B cell development. Cell *69*, 823-831.

Kitamura, D. and Rajewsky, K. (1992). Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion. Nature *356*, 154-156.

Kitamura, D., Roes, J., Kuhn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature *350*, 423-426.

Klug, C.A., Gerety, S.J., Shah, P.C., Chen, Y.Y., Rice, N.R., Rosenberg, N., and Singh, H. (1994). The v-abl tyrosine kinase negatively regulates NF-kappa B/Rel factors and blocks kappa gene transcription in pre-B lymphocytes. Genes Dev. *8*, 678-687.

Komori, T., Okada, A., Stewart, V., and Alt, F.W. (1993). Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. Science *261*, 1171-1175.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kontgen, F., Suss, G., Stewart, C., Steinmetz, M., and Bluethmann, H. (1993). Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. Int. Immunol. *5*, 957-964.

Kottmann, A.H., Brack, C., Eibel, H., and Kohler, G. (1992). A survey of protein-DNA interaction sites within the murine immunoglobulin heavy chain locus reveals a particularly complex pattern around the DQ52 element. Eur. J. Immunol. *22*, 2113-2120.

Kottmann, A.H., Zevnik, B., Welte, M., Nielsen, P.J., and Kohler, G. (1994). A second promoter and enhancer element within the immunoglobulin heavy chain locus. Eur. J. Immunol. *24*, 817-821.

Kouro, T., Medina, K.L., Oritani, K., and Kincade, P.W. (2001). Characteristics of early murine B-lymphocyte precursors and their direct sensitivity to negative regulators. Blood *97*, 2708-2715.

Kretschmer, K., Engel, H., and Weiss, S. (2002). Strong antigenic selection shaping the immunoglobulin heavy chain repertoire of B-1a lymphocytes in lambda 2(315) transgenic mice. Eur. J. Immunol. *32*, 2317-2327.

Kretschmer, K., Jungebloud, A., Stopkowicz, J., Kleinke, T., Hoffmann, R., and Weiss, S. (2003a). The selection of marginal zone B cells differs from that of B-1a cells. J. Immunol. *171*, 6495-6501.

Kretschmer, K., Jungebloud, A., Stopkowicz, J., Stoermann, B., Hoffmann, R., and Weiss, S. (2003b). Antibody repertoire and gene expression profile: implications for different developmental and functional traits of splenic and peritoneal B-1 lymphocytes. J. Immunol. *171*, 1192-1201.

Kroese, F.G., Butcher, E.C., Stall, A.M., Lalor, P.A., Adams, S., and Herzenberg, L.A. (1989). Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int. Immunol. *1*, 75-84.

Kruisbeek, A.M. (1999). Introduction: regulation of T cell development by the thymic microenvironment. Semin. Immunol. *11*, 1-2.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Kurioka, H., Kishi, H., Isshiki, H., Tagoh, H., Mori, K., Kitagawa, T., Nagata, T., Dohi, K., and Muraguchi, A. (1996). Isolation and characterization of a TATA-less promoter for the human RAG-1 gene. Mol. Immunol. *33*, 1059-1066.

Kwon, J., Imbalzano, A.N., Matthews, A., and Oettinger, M.A. (1998). Accessibility of nucleosomal DNA to V(D)J cleavage is modulated by RSS positioning and HMG1. Mol. Cell *2*, 829-839.

Kwon, J., Morshead, K.B., Guyon, J.R., Kingston, R.E., and Oettinger, M.A. (2000). Histone acetylation and hSWI/SNF remodeling act in concert to stimulate V(D)J cleavage of nucleosomal DNA. Mol. Cell *6*, 1037-1048.

Lacaud, G., Carlsson, L., and Keller, G. (1998). Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity. *9*, 827-838.

Lafaille, J.J., DeCloux, A., Bonneville, M., Takagaki, Y., and Tonegawa, S. (1989). Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. Cell *59*, 859-870.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. U. S. A *89*, 6232-6236.

Lalor, P.A., Herzenberg, L.A., Adams, S., and Stall, A.M. (1989a). Feedback regulation of murine Ly-1 B cell development. Eur. J. Immunol. *19*, 507-513.

Lalor, P.A., Stall, A.M., Adams, S., and Herzenberg, L.A. (1989b). Permanent alteration of the murine Ly-1 B repertoire due to selective depletion of Ly-1 B cells in neonatal animals. Eur. J. Immunol. *19*, 501-506.

Lam, K.P. and Rajewsky, K. (1999). B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. J. Exp. Med. *190*, 471-477.

Lam, K.P. and Stall, A.M. (1994). Major histocompatibility complex class II expression distinguishes two distinct B cell developmental pathways during ontogeny. J. Exp. Med. *180*, 507-516.

Landau, N.R., Schatz, D.G., Rosa, M., and Baltimore, D. (1987). Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol. Cell Biol. *7*, 3237-3243.

Lang, J., Arnold, B., Hammerling, G., Harris, A.W., Korsmeyer, S., Russell, D., Strasser, A., and Nemazee, D. (1997). Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose-dependent manner and promotes receptor editing in autoreactive, immature B cells. J. Exp. Med. *186*, 1513-1522.

Lauring, J. and Schlissel, M.S. (1999). Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. Mol. Cell Biol. *19*, 2601-2612.

Leclercq, L., Butkeraitis, P., and Reth, M. (1989). A novel germ-line JK transcript starting immediately upstream of JK1. Nucleic Acids Res. *17*, 6809-6819.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S., and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase cbeta-deficient mice. Science *273*, 788-791.

Lenardo, M., Pierce, J.W., and Baltimore, D. (1987). Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science *236*, 1573-1577.

Lenardo, M.J. and Baltimore, D. (1989). NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. Cell *58*, 227-229.

Lennon, G.G. and Perry, R.P. (1985). C mu-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5'-nontranslatable exon. Nature *318*, 475-478.

Lennon, G.G. and Perry, R.P. (1990). The temporal order of appearance of transcripts from unrearranged and rearranged Ig genes in murine fetal liver. J. Immunol. *144*, 1983-1987.

Lewandoski, M. (2001). Conditional control of gene expression in the mouse. Nat. Rev. Genet. *2*, 743-755.

Lewis, S., Gifford, A., and Baltimore, D. (1985). DNA elements are asymmetrically joined during the site-specific recombination of kappa immunoglobulin genes. Science *228*, 677-685.

Lewis, S.M. (1994). The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv. Immunol. *56*, 27-150.

Li, L., Milner, L.A., Deng, Y., Iwata, M., Banta, A., Graf, L., Marcovina, S., Friedman, C., Trask, B.J., Hood, L., and Torok-Storb, B. (1998). The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. Immunity. *8*, 43-55.

Li, Y.S., Hayakawa, K., and Hardy, R.R. (1993). The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. *178*, 951-960.

Li, Y.S., Wasserman, R., Hayakawa, K., and Hardy, R.R. (1996). Identification of the earliest B lineage stage in mouse bone marrow. Immunity. *5*, 527-535.

Li, Z., Otevrel, T., Gao, Y., Cheng, H.L., Seed, B., Stamato, T.D., Taccioli, G.E., and Alt, F.W. (1995). The XRCC4 gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination. Cell *83*, 1079-1089.

Li, Z., Woo, C.J., Iglesias-Ussel, M.D., Ronai, D., and Scharff, M.D. (2004). The generation of antibody diversity through somatic hypermutation and class switch recombination. Genes Dev. *18*, 1-11.

Lichtenstein, M., Keini, G., Cedar, H., and Bergman, Y. (1994). B cell-specific demethylation: a novel role for the intronic kappa chain enhancer sequence. Cell *76*, 913-923.

Lieber, M.R., Hesse, J.E., Mizuuchi, K., and Gellert, M. (1988). Lymphoid V(D)J recombination: nucleotide insertion at signal joints as well as coding joints. Proc. Natl. Acad. Sci. U. S. A *85*, 8588-8592.

Lin, W.C. and Desiderio, S. (1993). Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. Science *260*, 953-959.

Lin, W.C. and Desiderio, S. (1994). Cell cycle regulation of V(D)J recombination-activating protein RAG-2. Proc. Natl. Acad. Sci. U. S. A *91*, 2733-2737.

Lipshutz, R.J., Fodor, S.P., Gingeras, T.R., and Lockhart, D.J. (1999). High density synthetic oligonucleotide arrays. Nat. Genet. *21*, 20-24.

Lleo, A., Berezovska, O., Ramdya, P., Fukumoto, H., Raju, S., Shah, T., and Hyman, B.T. (2003). Notch1 competes with the amyloid precursor protein for gamma-secretase and down-regulates presenilin-1 gene expression. J. Biol. Chem. *278*, 47370-47375.

Lo, K., Landau, N.R., and Smale, S.T. (1991). LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. Mol. Cell Biol. *11*, 5229-5243.

Lobe, C.G. and Nagy, A. (1998). Conditional genome alteration in mice. Bioessays *20*, 200-208.

Lockhart, D.J., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E.L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. *14*, 1675-1680.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J. Exp. Med. *190*, 75-89.

Loffert, D., Ehlich, A., Muller, W., and Rajewsky, K. (1996). Surrogate light chain expression is required to establish immunoglobulin heavy chain allelic exclusion during early B cell development. Immunity. *4*, 133-144.

Longacre, A. and Storb, U. (2000). A novel cytidine deaminase affects antibody diversity. Cell *102*, 541-544.

Lowe, L.A., Yamada, S., and Kuehn, M.R. (2001). Genetic dissection of nodal function in patterning the mouse embryo. Development *128*, 1831-1843.

Lu, L.S., Tung, J., Baumgarth, N., Herman, O., Gleimer, M., Herzenberg, L.A., and Herzenberg, L.A. (2002). Identification of a germ-line pro-B cell subset that distinguishes the fetal/neonatal from the adult B cell development pathway. Proc. Natl. Acad. Sci. U. S. A *99*, 3007-3012.

Ludwig, D.L., Stringer, J.R., Wight, D.C., Doetschman, H.C., and Duffy, J.J. (1996). FLP-mediated site-specific recombination in microinjected murine zygotes. Transgenic Res. *5*, 385-395.

Ma, Y., Pannicke, U., Schwarz, K., and Lieber, M.R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell *108*, 781-794.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-2226.

Marshall, A.J., Wu, G.E., and Paige, G.J. (1996). Frequency of VH81x usage during B cell development: initial decline in usage is independent of Ig heavy chain cell surface expression. J. Immunol. *156*, 2077-2084.

Martensson, A., Argon, Y., Melchers, F., Dul, J.L., and Martensson, I.L. (1999). Partial block in B lymphocyte development at the transition into the pre-B cell receptor stage in Vpre-B1-deficient mice. Int. Immunol. *11*, 453-460.

Martensson, I.L., Rolink, A., Melchers, F., Mundt, C., Licence, S., and Shimizu, T. (2002). The pre-B cell receptor and its role in proliferation and Ig heavy chain allelic exclusion. Semin. Immunol. *14*, 335-342.

Marth, J.D. (1996). Recent advances in gene mutagenesis by site-directed recombination. J. Clin. Invest *97*, 1999-2002.

Martin, D., Huang, R.Q., LeBien, T., and Van Ness, B. (1991). Induced rearrangement of kappa genes in the BLIN-1 human pre-B cell line correlates with germline J-C kappa and V kappa transcription. J. Exp. Med. *173*, 639-645.

Martin, D.J. and Van Ness, B.G. (1990). Initiation and processing of two kappa immunoglobulin germ line transcripts in mouse B cells. Mol. Cell Biol. *10*, 1950-1958.

Martin, F. and Kearney, J.F. (2000a). B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". Immunol. Rev. *175*, 70-79.

Martin, F. and Kearney, J.F. (2000b). Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk. Immunity. *12*, 39-49.

Martin, F. and Kearney, J.F. (2001). B1 cells: similarities and differences with other B cell subsets. Curr. Opin. Immunol. *13*, 195-201.

Martin, F. and Kearney, J.F. (2002). Marginal-zone B cells. Nat. Rev. Immunol. *2*, 323-335.

Martin, F., Oliver, A.M., and Kearney, J.F. (2001). Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity. *14*, 617-629.

Maruyama, M., Lam, K.P., and Rajewsky, K. (2000). Memory B-cell persistence is independent of persisting immunizing antigen. Nature *407*, 636-642.

Mather, E.L. and Perry, R.P. (1983). Methylation status and DNase I sensitivity of immunoglobulin genes: changes associated with rearrangement. Proc. Natl. Acad. Sci. U. S. A *80*, 4689-4693.

Matsuda, M., Korn, B.S., Hammer, R.E., Moon, Y.A., Komuro, R., Horton, J.D., Goldstein, J.L., Brown, M.S., and Shimomura, I. (2001). SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. Genes Dev. *15*, 1206-1216.

Max, E.E. (1999). Immunoglobulins: Molecular Genetics. In *Fundamental immunology* (Edited by Paul W.E.), pp. 111-182, Lippincott-Raven, Philadelphia.

Max, E.E., Maizel, J.V., Jr., and Leder, P. (1981). The nucleotide sequence of a 5.5-kilobase DNA segment containing the mouse kappa immunoglobulin J and C region genes. J. Biol. Chem. *256*, 5116-5120.

McBlane, F. and Boyes, J. (2000). Stimulation of V(D)J recombination by histone acetylation. Curr. Biol. *10*, 483-486.

McCormack, W.T., Tjoelker, L.W., Carlson, L.M., Petryniak, B., Barth, C.F., Humphries, E.H., and Thompson, C.B. (1989). Chicken IgL gene rearrangement involves deletion of a circular episome and addition of single nonrandom nucleotides to both coding segments. Cell *56*, 785-791.

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J., and Maki, R.A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. *15*, 5647-5658.

McMurry, M.T. and Krangel, M.S. (2000). A role for histone acetylation in the developmental regulation of VDJ recombination. Science *287*, 495-498.

Mebius, R.E., Miyamoto, T., Christensen, J., Domen, J., Cupedo, T., Weissman, I.L., and Akashi, K. (2001). The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. J. Immunol. *166*, 6593-6601.

Melchers, F. and Rolink, A. (1999). B-Lymphocyte Development and Biology. In *Fundamental immunology* (Edited by Paul W.E.), pp. 183-224, Lippincott-Raven, Philadelphia.

Melchers, F., Rolink, A., Grawunder, U., Winkler, T.H., Karasuyama, H., Ghia, P., and Andersson, J. (1995). Positive and negative selection events during B lymphopoiesis. Curr. Opin. Immunol. *7*, 214-227.

Meyer, K.B., Sharpe, M.J., Surani, M.A., and Neuberger, M.S. (1990). The importance of the 3'-enhancer region in immunoglobulin kappa gene expression. Nucleic Acids Res. *18*, 5609-5615.

Meyers, E.N., Lewandoski, M., and Martin, G.R. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat. Genet. *18*, 136-141.

Milner, L.A. and Bigas, A. (1999). Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood *93*, 2431-2448.

Miyake, K., Medina, K., Ishihara, K., Kimoto, M., Auerbach, R., and Kincade, P.W. (1991). A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. J. Cell Biol. *114*, 557-565.

Molina, H., Holers, V.M., Li, B., Fung, Y., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R.W., and Chaplin, D.D. (1996). Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc. Natl. Acad. Sci. U. S. A *93*, 3357-3361.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869-877.

Monroe, R.J., Chen, F., Ferrini, R., Davidson, L., and Alt, F.W. (1999a). RAG2 is regulated differentially in B and T cells by elements 5' of the promoter. Proc. Natl. Acad. Sci. U. S. A *96*, 12713-12718.

Monroe, R.J., Seidl, K.J., Gaertner, F., Han, S., Chen, F., Sekiguchi, J., Wang, J., Ferrini, R., Davidson, L., Kelsoe, G., and Alt, F.W. (1999b). RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. Immunity. *11*, 201-212.

Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2001). Bipotential B-macrophage progenitors are present in adult bone marrow. Nat. Immunol. *2*, 83-88.

Moore, M.W., Durdik, J., Persiani, D.M., and Selsing, E. (1985). Deletions of kappa chain constant region genes in mouse lambda chain-producing B cells involve intrachromosomal DNA recombinations similar to V-J joining. Proc. Natl. Acad. Sci. U. S. A *82*, 6211-6215.

Morgan, B.P. (2000). The complement system: an overview. Methods Mol. Biol. *150*, 1-13.

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. *11*, 35-71.

Moshous, D., Callebaut, I., de Chasseval, R., Corneo, B., Cavazzana-Calvo, M., Le Deist, F., Tezcan, I., Sanal, O., Bertrand, Y., Philippe, N., Fischer, A., and de Villartay, J.P. (2001). Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. Cell *105*, 177-186.

Muller, B., Stappert, H., and Reth, M. (1990). A physical map and analysis of the murine C kappa-RS region show the presence of a conserved element. Eur. J. Immunol. *20*, 1409-1411.

Muller, W., Kuhn, R., and Rajewsky, K. (1991). Major histocompatibility complex class II hyperexpression on B cells in interleukin 4-transgenic mice does not lead to B cell proliferation and hypergammaglobulinemia. Eur. J. Immunol. *21*, 921-925.

Mundt, C., Licence, S., Shimizu, T., Melchers, F., and Martensson, I.L. (2001). Loss of precursor B cell expansion but not allelic exclusion in VpreB1/VpreB2 double-deficient mice. J. Exp. Med. *193*, 435-445.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell *102*, 553-563.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A.K., Pirity, M., and Rossant, J. (1998). Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. Curr. Biol. *8*, 661-664.

Nelson, K.J., Haimovich, J., and Perry, R.P. (1983). Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of micron and muS mRNA. Mol. Cell Biol. *3*, 1317-1332.

Nelson, K.J., Kelley, D.E., and Perry, R.P. (1985). Inducible transcription of the unrearranged kappa constant region locus is a common feature of pre-B cells and does not require DNA or protein synthesis. Proc. Natl. Acad. Sci. U. S. A *82*, 5305-5309.

Nelson, K.J., Mather, E.L., and Perry, R.P. (1984). Lipopolysaccharide-induced transcription of the kappa immunoglobulin locus occurs on both alleles and is independent of methylation status. Nucleic Acids Res. *12*, 1911-1923.

Nemazee, D. (2000). Receptor editing in B cells. Adv. Immunol. *74*, 89-126.

Nemazee, D.A. and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562-566.

Ng, H.H. and Bird, A. (1999). DNA methylation and chromatin modification. Curr. Opin. Genet. Dev. *9*, 158-163.

Nitschke, L., Carsetti, R., Ocker, B., Kohler, G., and Lamers, M.C. (1997). CD22 is a negative regulator of B-cell receptor signalling. Curr. Biol. *7*, 133-143.

Noben-Trauth, N., Kohler, G., Burki, K., and Ledermann, B. (1996). Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. Transgenic Res. *5*, 487-491.

Noguchi, M., Nakamura, Y., Russell, S.M., Ziegler, S.F., Tsang, M., Cao, X., and Leonard, W.J. (1993). Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science *262*, 1877-1880.

Norsworthy, P.J., Taylor, P.R., Walport, M.J., and Botto, M. (1999). Cloning of the mouse homolog of the 126-kDa human C1q/MBL/SP-A receptor, C1qR(p). Mamm. Genome *10*, 789-793.

Nussenzweig, A., Chen, C., da, C.S., V, Sanchez, M., Sokol, K., Nussenzweig, M.C., and Li, G.C. (1996). Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. Nature *382*, 551-555.

Nussenzweig, M.C. (1998). Immune receptor editing: revise and select. Cell *95*, 875-878.

Nutt, S.L., Heavey, B., Rolink, A.G., and Busslinger, M. (1999a). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature *401*, 556-562.

Nutt, S.L., Morrison, A.M., Dorfler, P., Rolink, A., and Busslinger, M. (1998). Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J. *17*, 2319-2333.

Nutt, S.L., Urbanek, P., Rolink, A., and Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. *11*, 476-491.

Nutt, S.L., Vambrie, S., Steinlein, P., Kozmik, Z., Rolink, A., Weith, A., and Busslinger, M. (1999b). Independent regulation of the two Pax5 alleles during B-cell development. Nat. Genet. *21*, 390-395.

O'Gorman, S., Fox, D.T., and Wahl, G.M. (1991). Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science *251*, 1351-1355.

O'Keefe, T.L., Williams, G.T., Davies, S.L., and Neuberger, M.S. (1996). Hyperresponsive B cells in CD22-deficient mice. Science *274*, 798-801.

Oberdoerffer, P., Otipoby, K.L., Maruyama, M., and Rajewsky, K. (2003). Unidirectional Cre-mediated genetic inversion in mice using the mutant loxP pair lox66/lox71. Nucleic Acids Res. *31*, e140.

Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., and Zinkernagel, R.M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. Science *286*, 2156-2159.

Oettinger, M.A., Schatz, D.G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science *248*, 1517-1523.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H., and Nishikawa, S. (1991). Expression and function of c-kit in hemopoietic progenitor cells. J. Exp. Med. *174*, 63-71.

Ogawa, M., ten Boekel, E., and Melchers, F. (2000). Identification of CD19(-)B220(+)c-Kit(+)Flt3/Flk-2(+)cells as early B lymphoid precursors before pre-B-I cells in juvenile mouse bone marrow. Int. Immunol. *12*, 313-324.

Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C., Myerson, D., and Bernstein, I.D. (2000). Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. Blood *95*, 2847-2854.

Ohmura, K., Kawamoto, H., Fujimoto, S., Ozaki, S., Nakao, K., and Katsura, Y. (1999). Emergence of T, B, and myeloid lineage-committed as well as multipotent hemopoietic progenitors in the aorta-gonad-mesonephros region of day 10 fetuses of the mouse. J. Immunol. *163*, 4788-4795.

Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. (1992). A transgenic model of autoimmune hemolytic anemia. J. Exp. Med. *175*, 71-79.

Oliver, A.M., Martin, F., Gartland, G.L., Carter, R.H., and Kearney, J.F. (1997). Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. Eur. J. Immunol. *27*, 2366-2374.

Oltz, E.M., Yancopoulos, G.D., Morrow, M.A., Rolink, A., Lee, G., Wong, F., Kaplan, K., Gillis, S., Melchers, F., and Alt, F.W. (1992). A novel regulatory myosin light chain gene distinguishes pre-B cell subsets and is IL-7 inducible. EMBO J. *11*, 2759-2767.

Otipoby, K.L., Andersson, K.B., Draves, K.E., Klaus, S.J., Farr, A.G., Kerner, J.D., Perlmutter, R.M., Law, C.L., and Clark, E.A. (1996). CD22 regulates thymus-independent responses and the lifespan of B cells. Nature *384*, 634-637.

Ouyang, H., Nussenzweig, A., Kurimasa, A., Soares, V.C., Li, X., Cordon-Cardo, C., Li, W., Cheong, N., Nussenzweig, M., Iliakis, G., Chen, D.J., and Li, G.C. (1997). Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination In vivo. J. Exp. Med. *186*, 921-929.

Pan, C., Baumgarth, N., and Parnes, J.R. (1999). CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. Immunity. *11*, 495-506.

Pandey, A., Ozaki, K., Baumann, H., Levin, S.D., Puel, A., Farr, A.G., Ziegler, S.F., Leonard, W.J., and Lodish, H.F. (2000). Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. Nat. Immunol. *1*, 59-64.

Papavasiliou, F., Casellas, R., Suh, H., Qin, X.F., Besmer, E., Pelanda, R., Nemazee, D., Rajewsky, K., and Nussenzweig, M.C. (1997). V(D)J recombination in mature B cells: a mechanism for altering antibody responses. Science *278*, 298-301.

Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., and Chan, A.C. (1999). Requirement for B cell linker protein (BLNK) in B cell development. Science *286*, 1949-1954.

Park, L.S., Martin, U., Garka, K., Gliniak, B., Di Santo, J.P., Muller, W., Largaespada, D.A., Copeland, N.G., Jenkins, N.A., Farr, A.G., Ziegler, S.F., Morrissey, P.J., Paxton, R., and Sims, J.E. (2000). Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. J. Exp. Med. *192*, 659-670.

Parker, D.C. (1993). T cell-dependent B cell activation. Annu. Rev. Immunol. *11*, 331-360.

Paull, T.T. (2001). New glimpses of an old machine. Cell *107*, 563-565.

Paulnock, D.M. (1992). Macrophage activation by T cells. Curr. Opin. Immunol. *4*, 344-349.

Pearson, W.R. and Lipman, D.J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U. S. A *85*, 2444-2448.

Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc. Natl. Acad. Sci. U. S. A *99*, 4489-4494.

Pelanda, R., Hobeika, E., Kurokawa, T., Zhang, Y., Kuppig, S., and Reth, M. (2002). Cre recombinase-controlled expression of the mb-1 allele. Genesis. *32*, 154-157.

Perlmutter, R.M., Kearney, J.F., Chang, S.P., and Hood, L.E. (1985). Developmentally controlled expression of immunoglobulin VH genes. Science *227*, 1597-1601.

Perry, R.P., Kelley, D.E., Coleclough, C., Seidman, J.G., Leder, P., Tonegawa, S., Matthyssens, G., and Weigert, M. (1980). Transcription of mouse kappa chain genes: implications for allelic exclusion. Proc. Natl. Acad. Sci. U. S. A *77*, 1937-1941.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B., and . (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J. Exp. Med. *180*, 1955-1960.

Petrenko, O., Beavis, A., Klaine, M., Kittappa, R., Godin, I., and Lemischka, I.R. (1999). The molecular characterization of the fetal stem cell marker AA4. Immunity. *10*, 691-700.

Picard, D. and Schaffner, W. (1984). Unrearranged immunoglobulin lambda variable region is transcribed in kappa-producing myelomas. EMBO J. *3*, 3031-3035.

Picard, D. and Schaffner, W. (1985). Cell-type preference of immunoglobulin kappa and lambda gene promoters. EMBO J. *4*, 2831-2838.

Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J. Biol. Chem. *274*, 305-315.

Pui, J.C., Allman, D., Xu, L., DeRocco, S., Karnell, F.G., Bakkour, S., Lee, J.Y., Kadesch, T., Hardy, R.R., Aster, J.C., and Pear, W.S. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity. *11*, 299-308.

Qian, Y., Santiago, C., Borrero, M., Tedder, T.F., and Clarke, S.H. (2001). Lupus-specific antiribonucleoprotein B cell tolerance in nonautoimmune mice is maintained by differentiation to B-1 and governed by B cell receptor signaling thresholds. J. Immunol. *166*, 2412-2419.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity. *10*, 547-558.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751-758.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A., Muller, W., Roes, J., and Schwenk, F. (1996). Conditional gene targeting. J. Clin. Invest *98*, 600-603.

Ramsden, D.A. and Wu, G.E. (1991). Mouse kappa light-chain recombination signal sequences mediate recombination more frequently than do those of lambda light chain. Proc. Natl. Acad. Sci. U. S. A *88*, 10721-10725.

Reap, E.A., Sobel, E.S., Cohen, P.L., and Eisenberg, R.A. (1993). Conventional B cells, not B-1 cells, are responsible for producing autoantibodies in lpr mice. J. Exp. Med. *177*, 69-78.

Reth, M., Wienands, J., and Schamel, W.W. (2000). An unsolved problem of the clonal selection theory and the model of an oligomeric B-cell antigen receptor. Immunol. Rev. *176*, 10-18.

Reth, M.G. and Alt, F.W. (1984). Novel immunoglobulin heavy chains are produced from DJH gene segment rearrangements in lymphoid cells. Nature *312*, 418-423.

Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A., Tezcan, I., Ersoy, F., Kayserili, H., Ugazio, A.G., Brousse, N., Muramatsu, M., Notarangelo, L.D., Kinoshita, K., Honjo, T., Fischer, A., and Durandy, A. (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell *102*, 565-575.

Rice, G.E. and Bevilacqua, M.P. (1989). An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science *246*, 1303-1306.

Rickert, R.C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature *376*, 352-355.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139-140.

Rolink, A., Grawunder, U., Haasner, D., Strasser, A., and Melchers, F. (1993a). Immature surface Ig+ B cells can continue to rearrange kappa and lambda L chain gene loci. J. Exp. Med. *178*, 1263-1270.

Rolink, A., Grawunder, U., Winkler, T.H., Karasuyama, H., and Melchers, F. (1994). IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. Int. Immunol. *6*, 1257-1264.

Rolink, A., Karasuyama, H., Grawunder, U., Haasner, D., Kudo, A., and Melchers, F. (1993b). B cell development in mice with a defective lambda 5 gene. Eur. J. Immunol. *23*, 1284-1288.

Rolink, A., Kudo, A., Karasuyama, H., Kikuchi, Y., and Melchers, F. (1991). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J. *10*, 327-336.

Rolink, A. and Melchers, F. (1996). B-cell development in the mouse. Immunol. Lett. *54*, 157-161.

Rolink, A., ten Boekel, E., Melchers, F., Fearon, D.T., Krop, I., and Andersson, J. (1996). A subpopulation of B220+ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. J. Exp. Med. *183*, 187-194.

Rolink, A.G., Andersson, J., and Melchers, F. (1998). Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. Eur. J. Immunol. *28*, 3738-3748.

Rolink, A.G., Nutt, S.L., Melchers, F., and Busslinger, M. (1999a). Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. Nature *401*, 603-606.

Rolink, A.G., ten Boekel, E., Yamagami, T., Ceredig, R., Andersson, J., and Melchers, F. (1999b). B cell development in the mouse from early progenitors to mature B cells. Immunol. Lett. *68*, 89-93.

Rolink, A.G., Tschopp, J., Schneider, P., and Melchers, F. (2002). BAFF is a survival and maturation factor for mouse B cells. Eur. J. Immunol. *32*, 2004-2010.

Rooney, S., Sekiguchi, J., Zhu, C., Cheng, H.L., Manis, J., Whitlow, S., DeVido, J., Foy, D., Chaudhuri, J., Lombard, D., and Alt, F.W. (2002). Leaky Scid phenotype associated with defective V(D)J coding end processing in Artemis-deficient mice. Mol. Cell *10*, 1379-1390.

Roschenthaler, F., Hameister, H., and Zachau, H.G. (2000). The 5' part of the mouse immunoglobulin kappa locus as a continuously cloned structure. Eur. J. Immunol. *30*, 3349-3354.

Roschenthaler, F., Kirschbaum, T., Heim, V., Kirschbaum, V., Schable, K.F., Schwendinger, J., Zocher, I., and Zachau, H.G. (1999). The 5' part of the mouse immunoglobulin kappa locus. Eur. J. Immunol. *29*, 2065-2071.

Roselaar, S.E. and Daugherty, A. (1998). Apolipoprotein E-deficient mice have impaired innate immune responses to Listeria monocytogenes in vivo. J. Lipid Res. *39*, 1740-1743.

Rucker, E.B., III, Dierisseau, P., Wagner, K.U., Garrett, L., Wynshaw-Boris, A., Flaws, J.A., and Hennighausen, L. (2000). Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. Mol. Endocrinol. *14*, 1038-1052.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K., Nakagami-Yamaguchi, E., Hamada, Y., Aizawa, S., and Hirai, H. (2003). Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity. *18*, 675-685.

Sakai, T., Johnson, K.J., Murozono, M., Sakai, K., Magnuson, M.A., Wieloch, T., Cronberg, T., Isshiki, A., Erickson, H.P., and Fassler, R. (2001). Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. Nat. Med. *7*, 324-330.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A *74*, 5463-5467.

Sato, S., Miller, A.S., Inaoki, M., Bock, C.B., Jansen, P.J., Tang, M.L., and Tedder, T.F. (1996a). CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity. *5*, 551-562.

Sato, S., Ono, N., Steeber, D.A., Pisetsky, D.S., and Tedder, T.F. (1996b). CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. J. Immunol. *157*, 4371-4378.

Sauer, B. and Henderson, N. (1989). Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. Nucleic Acids Res. *17*, 147-161.

Schable, K.F., Thiebe, R., Bensch, A., Brensing-Kuppers, J., Heim, V., Kirschbaum, T., Lamm, R., Ohnrich, M., Pourrajabi, S., Roschenthaler, F., Schwendinger, J., Wichelhaus, D., Zocher, I., and Zachau, H.G. (1999). Characteristics of the immunoglobulin Vkappa genes, pseudogenes, relics and orphons in the mouse genome. Eur. J. Immunol. *29*, 2082-2086.

Schatz, D.G., Oettinger, M.A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. Cell *59*, 1035-1048.

Schatz, D.G., Oettinger, M.A., and Schlissel, M.S. (1992). V(D)J recombination: molecular biology and regulation. Annu. Rev. Immunol. *10*, 359-383.

Schebesta, M., Heavey, B., and Busslinger, M. (2002a). Transcriptional control of B-cell development. Curr. Opin. Immunol. *14*, 216-223.

Schebesta, M., Pfeffer, P.L., and Busslinger, M. (2002b). Control of pre-BCR signaling by Pax5-dependent activation of the BLNK gene. Immunity. *17*, 473-485.

Schlissel, M. (2002a). Allelic exclusion of immunoglobulin gene rearrangement and expression: why and how? Semin. Immunol. *14*, 207-212.

Schlissel, M.S. (2002b). Does artemis end the hunt for the hairpin-opening activity in V(D)J recombination? Cell *109*, 1-4.

Schlissel, M.S. and Baltimore, D. (1989). Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell *58*, 1001-1007.

Schmidt, K.N., Hsu, C.W., Griffin, C.T., Goodnow, C.C., and Cyster, J.G. (1998). Spontaneous follicular exclusion of SHP1-deficient B cells is conditional on the presence of competitor wild-type B cells. J. Exp. Med. *187*, 929-937.

Schroeder, T., Kohlhof, H., Rieber, N., and Just, U. (2003). Notch signaling induces multilineage myeloid differentiation and up-regulates PU.1 expression. J. Immunol. *170*, 5538-5548.

Scott, E.W., Simon, M.C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573-1577.

Seibler, J., Zevnik, B., Kuter-Luks, B., Andreas, S., Kern, H., Hennek, T., Rode, A., Heimann, C., Faust, N., Kauselmann, G., Schoor, M., Jaenisch, R., Rajewsky, K., Kuhn, R., and Schwenk, F. (2003). Rapid generation of inducible mouse mutants. Nucleic Acids Res. *31*, e12.

Selsing, E., Miller, J., Wilson, R., and Storb, U. (1982). Evolution of mouse immunoglobulin lambda genes. Proc. Natl. Acad. Sci. U. S. A *79*, 4681-4685.

Serwe, M. and Sablitzky, F. (1993). V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer. EMBO J. *12*, 2321-2327.

Shimizu, T., Mundt, C., Licence, S., Melchers, F., and Martensson, I.L. (2002). VpreB1/VpreB2/lambda 5 triple-deficient mice show impaired B cell development but functional allelic exclusion of the IgH locus. J. Immunol. *168*, 6286-6293.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., and . (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855-867.

Shinkura, R., Tian, M., Smith, M., Chua, K., Fujiwara, Y., and Alt, F.W. (2003). The influence of transcriptional orientation on endogenous switch region function. Nat. Immunol. *4*, 435-441.

Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D.G. (1995). A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. Proc. Natl. Acad. Sci. U. S. A *92*, 6522-6526.

Shockett, P.E., Zhou, S., Hong, X., and Schatz, D.G. (2004). Partial reconstitution of V(D)J rearrangement and lymphocyte development in RAG-deficient mice expressing inducible, tetracycline-regulated RAG transgenes. Mol. Immunol. *40*, 813-829.

Sidman, C.L., Shultz, L.D., Hardy, R.R., Hayakawa, K., and Herzenberg, L.A. (1986). Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice. Science *232*, 1423-1425.

Sims, J.E., Williams, D.E., Morrissey, P.J., Garka, K., Foxworthe, D., Price, V., Friend, S.L., Farr, A., Bedell, M.A., Jenkins, N.A., Copeland, N.G., Grabstein, K., and Paxton, R.J. (2000). Molecular cloning and biological characterization of a novel murine lymphoid growth factor. J. Exp. Med. *192*, 671-680.

Snapper, C.M. and Finkelmann, F.D. (1999). Immunoglobulin Class switching. In *Fundamental immunology* (Edited by Paul W.E.), pp. 831-861, Lippincott-Raven, Philadelphia.

Solvason, N., Lehuen, A., and Kearney, J.F. (1991). An embryonic source of Ly1 but not conventional B cells. Int. Immunol. *3*, 543-550.

Souabni, A., Cobaleda, C., Schebesta, M., and Busslinger, M. (2002). Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. Immunity. *17*, 781-793.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503-517.

Staeheli, P., Danielson, P., Haller, O., and Sutcliffe, J.G. (1986). Transcriptional activation of the mouse Mx gene by type I interferon. Mol. Cell Biol. *6*, 4770-4774.

Stall, A.M., Wells, S.M., and Lam, K.P. (1996). B-1 cells: unique origins and functions. Semin. Immunol. *8*, 45-59.

Storb, U. and Arp, B. (1983). Methylation patterns of immunoglobulin genes in lymphoid cells: correlation of expression and differentiation with undermethylation. Proc. Natl. Acad. Sci. U. S. A *80*, 6642-6646.

Strasser, A., Rolink, A., and Melchers, F. (1989). One synchronous wave of B cell development in mouse fetal liver changes at day 16 of gestation from dependence to independence of a stromal cell environment. J. Exp. Med. *170*, 1973-1986.

Su, L.K. and Kadesch, T. (1990). The immunoglobulin heavy-chain enhancer functions as the promoter for I mu sterile transcription. Mol. Cell Biol. *10*, 2619-2624.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., Yoshida, H., and Nishikawa, S. (1993). Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. U. S. A *90*, 9125-9129.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., and Koyasu, S. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science *283*, 390-392.

Szollosi, J., Horejsi, V., Bene, L., Angelisova, P., and Damjanovich, S. (1996). Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J. Immunol. *157*, 2939-2946.

Taccioli, G.E., Amatucci, A.G., Beamish, H.J., Gell, D., Xiang, X.H., Torres Arzayus, M.I., Priestley, A., Jackson, S.P., Marshak, R.A., Jeggo, P.A., and Herrera, V.L. (1998). Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. Immunity. *9*, 355-366.

Takeda, S., Zou, Y.R., Bluethmann, H., Kitamura, D., Muller, U., and Rajewsky, K. (1993). Deletion of the immunoglobulin kappa chain intron enhancer abolishes kappa chain gene rearrangement in cis but not lambda chain gene rearrangement in trans. EMBO J. *12*, 2329-2336.

Tang, H., Zhang, X.Q., Naruse, T., Ohbo, K., and Suda, T. (2002). Expression and function of NJ-1 surface antigen in megakaryopoiesis. Biochem. Biophys. Res. Commun. *292*, 667-674.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K., and Tybulewicz, V.L. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature *374*, 467-470.

Tarlinton, D.M., McLean, M., and Nossal, G.J. (1995). B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur. J. Immunol. *25*, 3388-3393.

Tedder, T.F. and Engel, P. (1994). CD20: a regulator of cell-cycle progression of B lymphocytes. Immunol. Today *15*, 450-454.

Tedford, K., Nitschke, L., Girkontaite, I., Charlesworth, A., Chan, G., Sakk, V., Barbacid, M., and Fischer, K.D. (2001). Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat. Immunol. *2*, 548-555.

ten Boekel, E., Melchers, F., and Rolink, A. (1995). The status of Ig loci rearrangements in single cells from different stages of B cell development. Int. Immunol. *7*, 1013-1019.

ten Boekel, E., Melchers, F., and Rolink, A.G. (1998). Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression. Immunity. *8*, 199-207.

Terkeltaub, R.A., Dyer, C.A., Martin, J., and Curtiss, L.K. (1991). Apolipoprotein (apo) E inhibits the capacity of monosodium urate crystals to stimulate neutrophils. Characterization of intraarticular apo E and demonstration of apo E binding to urate crystals in vivo. J. Clin. Invest *87*, 20-26.

Thai, T.H., Purugganan, M.M., Roth, D.B., and Kearney, J.F. (2002). Distinct and opposite diversifying activities of terminal transferase splice variants. Nat. Immunol. *3*, 457-462.

Thiebe, R., Schable, K.F., Bensch, A., Brensing-Kuppers, J., Heim, V., Kirschbaum, T., Mitlohner, H., Ohnrich, M., Pourrajabi, S., Roschenthaler, F., Schwendinger, J., Wichelhaus, D., Zocher, I., and Zachau, H.G. (1999). The variable genes and gene families of the mouse immunoglobulin kappa locus. Eur. J. Immunol. *29*, 2072-2081.

Thompson, C.B. (1995). New insights into V(D)J recombination and its role in the evolution of the immune system. Immunity. *3*, 531-539.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature *302*, 575-581.

van Gent, D.C., Mizuuchi, K., and Gellert, M. (1996). Similarities between initiation of V(D)J recombination and retroviral integration. Science *271*, 1592-1594.

Van Ness, B.G., Weigert, M., Coleclough, C., Mather, E.L., Kelley, D.E., and Perry, R.P. (1981). Transcription of the unrearranged mouse C kappa locus: sequence of the initiation region and comparison of activity with a rearranged V kappa-C kappa gene. Cell *27*, 593-602.

Varnum-Finney, B., Purton, L.E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K.A., Le, R., I, Mann, R., Gray, G., Artavanis-Tsakonas, S., and Bernstein, I.D. (1998). The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. Blood *91*, 4084-4091.

Villey, I., Caillol, D., Selz, F., Ferrier, P., and de Villartay, J.P. (1996). Defect in rearrangement of the most 5' TCR-J alpha following targeted deletion of T early alpha (TEA): implications for TCR alpha locus accessibility. Immunity. *5*, 331-342.

Vos, Q., Lees, A., Wu, Z.Q., Snapper, C.M., and Mond, J.J. (2000). B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. Immunol. Rev. *176*, 154-170.

Vosshenrich, C.A., Cumano, A., Muller, W., Di Santo, J.P., and Vieira, P. (2003). Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development. Nat. Immunol. *4*, 773-779.

Walker, L., Carlson, A., Tan-Pertel, H.T., Weinmaster, G., and Gasson, J. (2001). The notch receptor and its ligands are selectively expressed during hematopoietic development in the mouse. Stem Cells *19*, 543-552.

Wang, D., Feng, J., Wen, R., Marine, J.C., Sangster, M.Y., Parganas, E., Hoffmeyer, A., Jackson, C.W., Cleveland, J.L., Murray, P.J., and Ihle, J.N. (2000). Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. *13*, 25-35.

Wang, J.H., Avitahl, N., Cariappa, A., Friedrich, C., Ikeda, T., Renold, A., Andrikopoulos, K., Liang, L., Pillai, S., Morgan, B.A., and Georgopoulos, K. (1998). Aiolos regulates B cell activation and maturation to effector state. Immunity. *9*, 543-553.

Wang, Y., Spatz, M.K., Kannan, K., Hayk, H., Avivi, A., Gorivodsky, M., Pines, M., Yayon, A., Lonai, P., and Givol, D. (1999). A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. Proc. Natl. Acad. Sci. U. S. A *96*, 4455-4460.

Wasserman, R., Li, Y.S., Shinton, S.A., Carmack, C.E., Manser, T., Wiest, D.L., Hayakawa, K., and Hardy, R.R. (1998). A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. J. Exp. Med. *187*, 259-264.

Watanabe, N., Nisitani, S., Ikuta, K., Suzuki, M., Chiba, T., and Honjo, T. (1999). Expression levels of B cell surface immunoglobulin regulate efficiency of allelic exclusion and size of autoreactive B-1 cell compartment. J. Exp. Med. *190*, 461-469.

Watts, C. and Powis, S. (1999). Pathways of antigen processing and presentation. Rev. Immunogenet. *1*, 60-74.

Wayne, J., Suh, H., Misulovin, Z., Sokol, K.A., Inaba, K., and Nussenzweig, M.C. (1994a). A regulatory role for recombinase activating genes, RAG-1 and RAG-2, in T cell development. Immunity. *1*, 95-107.

Wayne, J., Suh, H., Sokol, K.A., Petrie, H.T., Witmer-Pack, M., Edelhoff, S., Disteche, C.M., and Nussenzweig, M.C. (1994b). TCR selection and allelic exclusion in RAG transgenic mice that exhibit abnormal T cell localization in lymph nodes and lymphatics. J. Immunol. *153*, 5491-5502.

Weinmaster, G. (2000). Notch signal transduction: a real rip and more. Curr. Opin. Genet. Dev. *10*, 363-369.

Whitehurst, C.E., Chattopadhyay, S., and Chen, J. (1999). Control of V(D)J recombinational accessibility of the D beta 1 gene segment at the TCR beta locus by a germline promoter. Immunity. *10*, 313-322.

Willerford, D.M., Swat, W., and Alt, F.W. (1996). Developmental regulation of V(D)J recombination and lymphocyte differentiation. Curr. Opin. Genet. Dev. *6*, 603-609.

Wilson, A., Ferrero, I., MacDonald, H.R., and Radtke, F. (2000). Cutting edge: an essential role for Notch-1 in the development of both thymus-independent and -dependent T cells in the gut. J. Immunol. *165*, 5397-5400.

Wilson, A., MacDonald, H.R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J. Exp. Med. *194*, 1003-1012.

Wineman, J.P., Gilmore, G.L., Gritzmacher, C., Torbett, B.E., and Muller-Sieburg, C.E. (1992). CD4 is expressed on murine pluripotent hematopoietic stem cells. Blood *80*, 1717-1724.

Winkler, T.H., Melchers, F., and Rolink, A.G. (1995). Interleukin-3 and interleukin-7 are alternative growth factors for the same B-cell precursors in the mouse. Blood *85*, 2045-2051.

Wither, J.E., Roy, V., and Brennan, L.A. (2000). Activated B cells express increased levels of costimulatory molecules in young autoimmune NZB and (NZB x NZW)F(1) mice. Clin. Immunol. *94*, 51-63.

Witt, C.M., Won, W.J., Hurez, V., and Klug, C.A. (2003). Notch2 haploinsufficiency results in diminished B1 B cells and a severe reduction in marginal zone B cells. J. Immunol. *171*, 2783-2788.

Wodicka, L., Dong, H., Mittmann, M., Ho, M.H., and Lockhart, D.J. (1997). Genome-wide expression monitoring in Saccharomyces cerevisiae. Nat. Biotechnol. *15*, 1359-1367.

Wolniak, K.L., Shinall, S.M., and Waldschmidt, T.J. (2004). The germinal center response. Crit Rev. Immunol. *24*, 39-65.

Xu, X., Li, C., Garrett-Beal, L., Larson, D., Wynshaw-Boris, A., and Deng, C.X. (2001). Direct removal in the mouse of a floxed neo gene from a three-loxP conditional knockout allele by two novel approaches. Genesis. *30*, 1-6.

Xu, Y., Davidson, L., Alt, F.W., and Baltimore, D. (1996). Deletion of the Ig kappa light chain intronic enhancer/matrix attachment region impairs but does not abolish V kappa J kappa rearrangement. Immunity. *4*, 377-385.

Yamagami, T., ten Boekel, E., Andersson, J., Rolink, A., and Melchers, F. (1999). Frequencies of multiple IgL chain gene rearrangements in single normal or kappaL chain-deficient B lineage cells. Immunity. *11*, 317-327.

Yancopoulos, G.D. and Alt, F.W. (1985). Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell *40*, 271-281.

Yancopoulos, G.D., Blackwell, T.K., Suh, H., Hood, L., and Alt, F.W. (1986). Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell *44*, 251-259.

Yancopoulos, G.D., Desiderio, S.V., Paskind, M., Kearney, J.F., Baltimore, D., and Alt, F.W. (1984). Preferential utilization of the most JH-proximal VH gene segments in pre-B-cell lines. Nature *311*, 727-733.

Yarnell, S.H., Landree, M.A., Qiu, J.X., Kale, S.B., and Roth, D.B. (2001). Joining-deficient RAG1 mutants block V(D)J recombination in vivo and hairpin opening in vitro. Mol. Cell *7*, 65-75.

Yother, J., Forman, C., Gray, B.M., and Briles, D.E. (1982). Protection of mice from infection with Streptococcus pneumoniae by anti-phosphocholine antibody. Infect. Immun. *36*, 184-188.

Yu, K., Chedin, F., Hsieh, C.L., Wilson, T.E., and Lieber, M.R. (2003). R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat. Immunol. *4*, 442-451.

Yu, W., Misulovin, Z., Suh, H., Hardy, R.R., Jankovic, M., Yannoutsos, N., and Nussenzweig, M.C. (1999a). Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5' of RAG2. Science *285*, 1080-1084.

Yu, W., Nagaoka, H., Jankovic, M., Misulovin, Z., Suh, H., Rolink, A., Melchers, F., Meffre, E., and Nussenzweig, M.C. (1999b). Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature *400*, 682-687.

Zabel, M.D., Weis, J.J., and Weis, J.H. (1999). Lymphoid transcription of the murine CD21 gene is positively regulated by histone acetylation. J. Immunol. *163*, 2697-2703.

Zarrin, A.A., Fong, I., Malkin, L., Marsden, P.A., and Berinstein, N.L. (1997). Cloning and characterization of the human recombination activating gene 1 (RAG1) and RAG2 promoter regions. J. Immunol. *159*, 4382-4394.

Zheng, B., Mills, A.A., and Bradley, A. (2001). Introducing defined chromosomal rearrangements into the mouse genome. Methods *24*, 81-94.

Zhu, C., Bogue, M.A., Lim, D.S., Hasty, P., and Roth, D.B. (1996). Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. Cell *86*, 379-389.

Zou, Y.R., Takeda, S., and Rajewsky, K. (1993). Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa. EMBO J. *12*, 811-820.

8. Abbreviations

AID	activation-induced cytidine deaminase
AmpR	ampicillin resistance gene
aRNA	antisense RNA
BAC	bacterial artificial chromosome
BCR	B cell receptor
BM	bone marrow
bp	base pairs
C	constant region gene segment
CD	cluster of differentiation
cDNA	complementary RNA
CDR	complementarity-determining region
CDS	coding sequence
CIAP	calf intestine alkaline phosphatase
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSR	class switch recombination
D	diversity gene segment
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DTA	diphtheria toxin A
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
EF	embryonic feeder
e.g.	*exempli gratia*

ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
EST	expressed sequence tag
et al.	*et alii*
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FL	fetal liver
FO	follicular
FTOC	fetal thymic organ culture
FWR	frame work region
H	heavy
HEL	hen egg lysozyme
HSC	hematopoietic stem cell
HSV	herpes simplex virus
i.e.	*id est*
IFN	interferon
Ig	immunoglobulin
IL	interleukin
J	joining gene segment
L	leader
L	light
LB	Luria Bertani
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAR	matrix attachment regions
MCS	multiple cloning site
MHC	major histocompatibility complex

mmc	mitomycin C
mRNA	messenger RNA
MZ	marginal zone
N	non-templated
NeoR	neomycin resistance gene
NHEJ	non-homologous DNA end-joining
NK	natural killer (cell)
NZB/NZW	New Zealand Black/New Zealand White
P	palindromic
PBS	phosphate buffered saline
PC	phosphorylcholine
PCR	polymerase chain reaction
pre	precursor
pro	progenitor
RACE	rapid amplification of cDNA ends
Rag	recombination activating gene
RNA	ribonucleic acid
RS	recombining sequence
RSS	recombination signal sequence
RT	reverse transcription
RT	room temperature
S	switch
scid	severe combined immunodeficiency
SDS	sodiumdodecylsulphate
sIg	surface immunoglobulin
sIgM	surface IgM
SL	surrogate light (chain)

SLE	systemic lupus erythematosus
SPF	specific pathogen free
TAE	Tris-acetic acid-EDTA
TCR	T cell receptor
TD	T cell dependent
TdT	terminal deoxynucleotidyl transferase
TEA	T early alpha
TI	T cell independent
TK	thymidine kinase
TSLP	thymic stromal-derived lymphopoietin
UTR	untranslated region
UV	ultraviolet
V	variable region gene segment
v/v	volume per volume
w/v	weight per volume

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